BIOLOGICALLY ACTIVE LIPIDS AND PLATELET FUNCTION

by

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Statement in terms of Ph.D. Regulation 2.4.15 of the Postgraduate Regulations of the University of Edinburgh.

I declare that this thesis was composed by myself and that all the experimental work described herein was performed by myself with the exception of the diabetic studies in chapter 6. These were carried out in collaboration with Dr. R.A. Armstrong who performed the collagen aggregation studies and the cyclic AMP/TXB_2 assays.

Dr. R.L. Jones

Paulina Tymkewycz

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Some of the results presented in this thesis have been published.

ARMSTRONG, R., JONES, R.L. and TYMKEWYCZ, P.M.
The nature of Arachidonic Acid-Induced Aggregation in Rat Platelets.
Br.J.Pharmac. (1985) 84, 147P.

CLARKE, B.F., COLLIER, A. and TYMKEWYCZ, P.M.
Changes in platelet function in diabetes mellitus with improvement of glycaemic control.
Br.J.Pharmac. (1985) 86, 795P.

COLLIER, A., TYMKEWYCZ, P.M., ARMSTRONG, R., YOUNG, R.J., JONES, R.L. and CLARKE, B.F.
Platelet receptor sensitivity and thromboxane production in diabetics with proliferative and background retinopathy.
Diabetologia (1986) 29, 471-474.

JONES, R.L., TYMKEWYCZ, P.M. and WILSON, N.H.
Characteristics of thromboxane receptors in platelets from man, rabbit and rat.
Abstract for the VIth International Conference on Prostaglandins, Florence 1986.

JONES, R.L., TYMKEWYCZ, P.M. and WILSON, N.H.
Differences in antagonist but not agonist potencies on the thromboxane-sensitive systems of human rat and rabbit platelets.
Abstract for B.P.S. Meeting, Dec.1986.

CONVENTIONS AND NOMENCLATURE

Trivial names of prostaglandins and their analogues have been used throughout this thesis. The systematic names for some of the C20 prostaglandins and analogues referred to in this thesis are shown below and those not listed may be deduced from these examples.

Trivial name	Systematic name
Prostaglandin H_2 (PGH_2)	15(S)-Hydroxy-9α,11α-peroxidoprosta-5*cis*, 13 *trans*-dienoic acid
Prostacyclin (PGI_2)	11α,15(S)-Dihydroxy-9-deoxy-6,9-epoxy-5Z,13-*trans* prostadienoic acid.
11,9-epoxymethano PGH_2 (11,9-emPGH_2)	15(S)Hydroxy-11α,9α(epoxymethano) prostadienoic acid
EP171	w-tetranor-16-p-fluorophenoxy-9α,11α-oxa-10a-homoPGH_2
STA_2	9,11-Epithio-11,12-methano TXA_2

In drawing of chemical structures, stereochemistry is not implied but a thickened or dotted line denotes a substituent located respectively above or below the plane of the paper.

ABSTRACT OF THESIS

Platelets are circulating quiescent cells which play an important role in haemostasis; following blood vessel injury, platelets adhere to the damaged vessel, aggregate and form a haemostatic plug which diminishes or arrests the haemorrhage. In addition the platelet has been shown to have an important part in the pathogenesis of thromboembolic disorders. Upon activation platelets release arachidonic acid from membrane phospholipids, conversion of which results in the production of prostaglandins and thromboxanes. The prostaglandin endoperoxides and thromboxane A_2 are potent platelet aggregating agents and mediators of the platelet release reaction. This thesis is primarily concerned with platelet function and several aspects have been studied.

(i) An investigation into the modes of action of arachidonic acid in rat platelets was carried out, since controversy exists as to the precise mediators of aggregation in this species. Over a range of arachidonic acid concentrations three types of response were evoked in washed rat platelet suspensions; a thromboxane-dependent irreversible aggregation at low concentrations; a suppression of aggregation associated with subsequent inhibition of other aggregating agents at intermediate concentrations; and an apparent irreversible aggregation associated with platelet lysis at high concentrations. The mechanisms underlying each of these responses were investigated.

(ii) A second aspect concerned the pharmacological characterization of the thromboxane-sensitive systems mediating aggregation of human, rat and rabbit platelets. The relative potencies of a range of thromboxane agonists were similar in the 3 species, yet when antagonists were studied the rabbit platelet thromboxane-receptor was consistently less sensitive to their blocking action.

(iii) The final section is concerned with platelet disfunction in two pathological conditions, diabetes and hypothyroidism. In the former condition an increase in platelet activity has been shown to be intimately related to enhanced thromboxane A_2 activity. This may simply reflect an increase in thromboxane A_2 production from endogenous arachidonic acid, although improved receptor coupling or an increase in the number of receptors may contribute.

In one study, platelet sensitivity to collagen and the stable thromboxane mimetic, 11,9epoxymethano PGH_2 was compared between control subjects and diabetics. An increased sensitivity to the thromboxane mimetic was observed in the group of diabetics with proliferative retinopathy and in contrast to the findings in the literature there was no apparent difference in sensitivity to collagen. In another study platelet sensitivity to the aggregating agents ADP and 11,9epoxymethano PGH_2 was measured in a group of poorly controlled diabetics who underwent short-term improvement of glycaemic control. At the end of the six month period, there was no detectable change in their sensitivity to aggregating agents ADP and 11,9emPGH_2 yet a marked increase in responsiveness to the inhibitory prostaglandin, Iloprost was observed.

Hypothyroidism like diabetes is associated with microvascular complications. A study was carried out on a group of hypothyroid patients, whose platelet sensitivity was measured at the beginning and the end of a six month period of hormone replacement. Platelet sensitivity was increased to a slight extent following hormone replacement therapy but a marked fall in cyclic AMP levels from plasma was found. This could suggest that improvement of the hormone deficiency may not necessarily be in favour of improving microvascular disorders.

LIST OF FIGURES

LIST OF TABLES

CONTENTS PAGE NUMBER

CHAPTER 1

General Introduction

Platelets are circulating anucleate cells playing a quiescent role under normal physiological conditions. Upon injury to the vessel wall circulating platelets adhere to the damaged vessel forming an aggregate or heamostatic plug which diminishes or arrests the haemorrhage. This interaction of platelets with the vessel wall demonstrates one physiological function of the platelets, and much clinical and experimental evidence has demonstrated that a deficiency or defect in circulating platelets is often associated with "spontaneous" haemorrhage from small vessels suggesting that platelets are in some way essential for the functional integrity of the blood vessels.

Platelets may play a pathological role in the formation of intravascular aggregates since it has been shown that occlusive thrombi in arteries damaged by atherosclerosis consist mainly of platelets, (Davis and Thomas, 1981). A process of activation is an essential prerequisite for adhesion and aggregation, inferred from the non-reactivity of normal circulating platelets. Until recently it was generally assumed that the process of aggregation depended upon the adhesion of platelets to collagen. Collagen lies beneath the endothelial cells that form the innermost layer of the vessel wall and is exposed when the vessel is damaged. Adhering platelets undergo aggregation and secrete the contents of intracellular granules in a process called the release reaction. The secreted materials include coagulation factors, vasoconstrictors and aggregating agents and these substances promote the recruitment of additional platelets to the aggregate.

More recently it has been demonstrated under experimental conditions that platelets still retain their ability to aggregate when no collagen is present (Born & Kratzner, 1984). It has been suggested that in regions of abnormal blood flow, red blood cells may release ADP which leads to the activation and aggregation of platelets (Born & Wehmeier, 1979).

Furthermore, the presence of fibrin in the thrombus, indicates that thrombin is present at the site of the damaged vessel and thrombin too is a potent aggregating agent.

It is probable therefore that no single agent is responsible for the initiation of platelet aggregation 'in vivo'. The initiation and perhaps the growth of thrombi from platelet aggregates may be influenced by products of the arachidonic acid (AA) pathway (Fig.1.1). Thromboxane A_2 (TXA_2) a metabolite of AA, synthesized within and released from the platelet, is a potent stimulator of platelet aggregation and constrictor of blood vessels. Prostacyclin, another AA metabolite is produced by endothelial cells and has opposing actions to TXA_2, whereby, it inhibits aggregation and dilates blood vessels. A balance between PGI_2 and TXA_2 may therefore prevent thrombus and haemostatic plug formation and in fact it has been suggested that an imbalance in the PGI_2-TXA_2 system may be related to certain pathological conditions; arterial thrombosis (Lagarde and Dechavanne, 1977), diabetes (Harrison et al, 1978; Johnson et al, 1978) and thrombocytopenic purpura (Remuzzi et al, 1980; Cocchetto et al, 1981). Prostacyclin synthetase, the enzyme which converts prostaglandin endoperoxides (PGG_2 and PGH_2) to PGI_2,

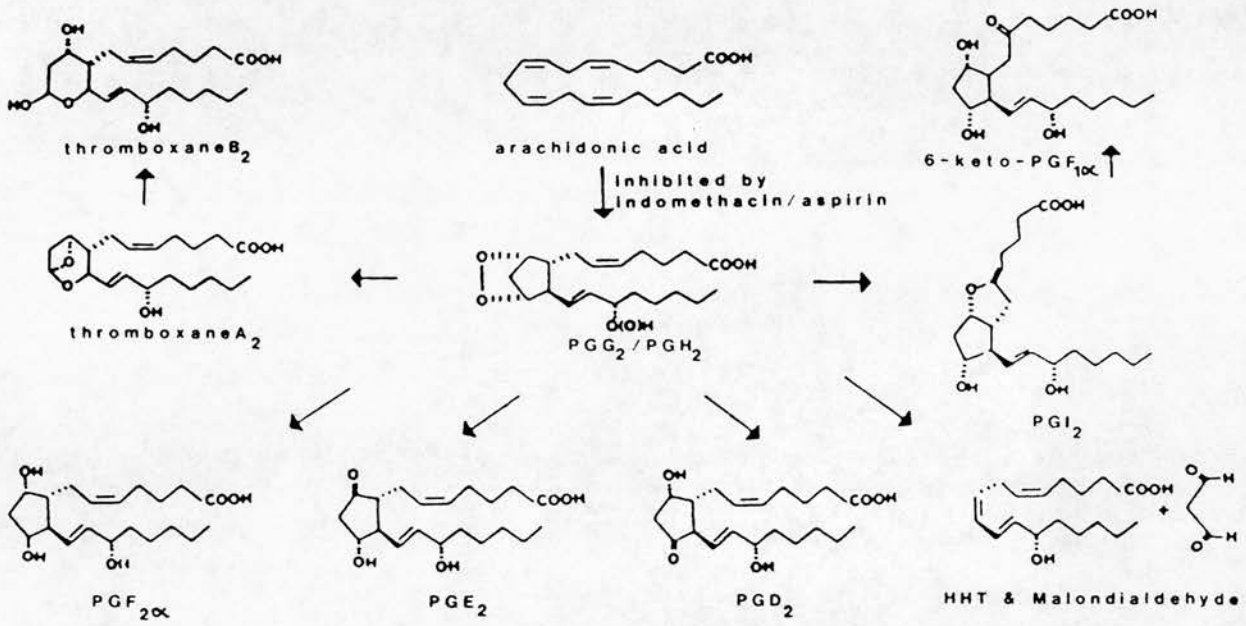


Figure 1.1 The major metabolites of arachidonic acid.

is most highly concentrated in the intimal surface of the blood vessel and progressively decreases towards the adventitial surface (Moncada et al, 1977). Damaged endothelium may have a reduced capacity for PGI_2 production, thus allowing platelet deposition and aggregation to occur more readily. A fall in PGI_2 production at the site of the damaged vessel may be a contributory factor to the initiation of thrombus formation.

Before one can elucidate the mechanisms underlying platelet activation, it is necessary to have an understanding of the roles of the intracellular platelet ultra structures. Platelets contain extremely labile microtubules, protein structures which are sensitive to calcium ion fluxes, low temperature and pressure changes. A marginal ring of microtubules has been identified in platelets of most species studied so far and following platelet stimulation, the ring of microtubules reduces in diameter, contracting towards the centre of the cell, apparently concentrating the granular organelles within a central cytoplasmic zone bounded by the microtubule bundle (Nachmias et al, 1977). The microtubules in the activated platelet are then seen in a newly polymerised configuration which cause local activation of the contractile system giving rise to pseudopodia and spikes which are observed in the course of shape change. It is thought that the disassembly of the circumferential ring of microtubules activates the contractile system which subsequently gives rise to the pseudopods and spikes. The formation of these may facilitate aggregation and secretion by increasing the opportunity of cell-cell contact. The breakdown of the circumferential ring of microtubules has been proposed to be due to local increases in

calcium ions restricted to the submembraneous region (Le Breton et al, 1976). (By employing low concentration of chlortetracycline as a fluorescent probe for membrane calcium, a redistribution of calcium away from membrane sites during shape change was demonstrated). It was generally accepted that cytoplasmic calcium fluctuations played a central role in the initiation of platelet activation and the shape change response was found to be associated with elevations in cytosolic free calcium, which have proved to be insufficient to trigger aggregation or secretion. However, more recently, the shape change response induced by a number of agents such as thrombin (low concentrations), PAF-acether, ADP and adrenaline has been found to occur without detectable changes in cytosolic free calcium levels (indicated by quin-2) (Rink et al, 1982; Hallam et al, 1984A; 1984B).

Higher concentrations of the above agonists will subsequently induce platelets to aggregate and primary aggregation follows platelet shape change when platelets are stimulated with agents not sufficiently potent enough to promote second phase aggregation. Primary aggregation reflects the exposure of fibrinogen receptors on the platelet surface and the interaction of fibrinogen subsequently enhances cell-cell contact (Mustard et al, 1975). This response requires a lower cytosolic calcium concentration than that necessary to induce secondary wave aggregation and may in fact involve a different pool of bound calcium ions (Charo et al, 1976; Le Breton and Dinerstein, 1977). It has been suggested that extracellular calcium ions are essential for the primary response since in the presence of the chelating agent EGTA, primary aggregation is

suppressed. Under most circumstances, primary aggregation spontaneously reverses whereby platelets retain their discoid form and this occurs if the stimulus is not sufficient to induce endogenous thromboxane synthesis and the release reaction (Mustard et al, 1975).

In the presence of physiological calcium concentrations, ADP only induces a readily reversible platelet aggregatory response (no formation of TXA_2 or the release of granule contents). However, in a medium with a low calcium ion concentrations such as citrated plasma, high concentrations of ADP (1-2μM) induces two phases of aggregation in human platelets. The second phase is associated with activation of the arachidonate pathway and the release reaction (Macfarlane et al, 1975; Mustard et al, 1975). This effect is species specific since rabbit platelets never produce secondary wave aggregation in response to ADP regardless of the medium (Joist et al, 1974; Macmillan, 1970). This observation would therefore explain why 'in vivo' ADP only induces aggregation which is readily reversible in most species and it should be emphasised that ADP induction of the release reaction in some species is really only an artefact of the 'in vitro' system. Human platelets prepared with other anticoagulants such as hirudin and exposed to physiological calcium concentrations only demonstrate primary aggregation waves (Kinlough-Rathbone et al, 1983). These artefactual effects should be taken into account when considering the mode of ADP induced aggregation. Essentially the primary aggregation response results from a direct effect of agonists at the platelet surface and it is a process which is not affected by inhibitors of the cyclo-oxygenase

pathway. Secondary aggregation occurs as a consequence of the release reaction and/or endogenous TXA_2 synthesis. During the release reaction the contents of platelet storage vesicles are released from the platelet.

Platelet contains 3 types of storage vesicles; the dense granules containing ADP, 5HT, ATP, calcium, antiplasmin and inorganic phosphates; alpha granules containing fibrinogen, β-thromboglobulin cell growth factor and platelet factor 4; and platelet lysozomes. The movement of calcium across platelet membranes may be important in the release process since calcium ionophores can induce such an effect (Feinman and Detwiler, 1974) and calcium antagonists have been found to inhibit release (Charo et al, 1976). The movement of calcium into the cytoplasm may in fact exert an effect on the platelet microtubules which move towards the centre of the cell in a contractile wave during the release reaction (White, 1971). In addition calcium-dependent activation of platelet actino-myosin is thought to be involved (Haslam and Lynham, 1977; Daniel et al, 1977; Bennet et al, 1977). Actino-myosin is a protein complex, phosphorylation of which reaches a maximum before the completion of secondary aggregation. Phosphorylation is followed by a slow dephosphorylation. No increase in phosphorylation is observed when aggregation without secretion occurs and it seems therefore that phosphorylation is a prerequisite to the initiation of secretion (Bennet et al, 1977). The protein-actin complex has been postulated to be involved in vesicular fusion of dense bodies with the plasma-membrane, whereby an opening is formed through which the contents are expelled (Daniel et al, 1981).

The formation of endogenous TXA_2, during secondary wave aggregation occurs when arachidonate is released from membrane phospholipids. The role of endogenous TXA_2 in platelet aggregation was discovered in the early seventies from the use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin (Ferreira et al, 1971; Smith and Willis, 1971; Vane 1971). These drugs inhibit the prostaglandin synthetase (cyclo-oxygenase) enzyme and were important factors in the elucidation of platelet aggregation mechanisms.

Agents that can induce secondary wave aggregation include thrombin, collagen, PAF and TXA_2. They do so through one or both of the following mechanisms; (A) by activation of the phospholipase enzymes which cleave AA from membrane phospholipids. AA is then converted to the prostaglandin endoperoxides (PGG_2 and PGH_2) and TXA_2. (These enzymes are inactivated by free radicals formed during the reduction of PGG_2 to PGH_2 (Samuelsson et al, 1975; Smith and Lands, 1972)) and/or (B) by directly triggering the release of ADP and 5HT from platelet dense granules.

ADP, 5HT and TXA_2 potentiate aggregation by either recruiting other platelets or in the case of TXA_2 trigger further release, and it is these events which induce the second phase of aggregation. In addition, ADP, 5HT and TXA_2 may act synergistically with weak aggregating agents such as ADP or adrenaline (Kinlough-Rathbone et al, 1977; Packham et al, 1977A).

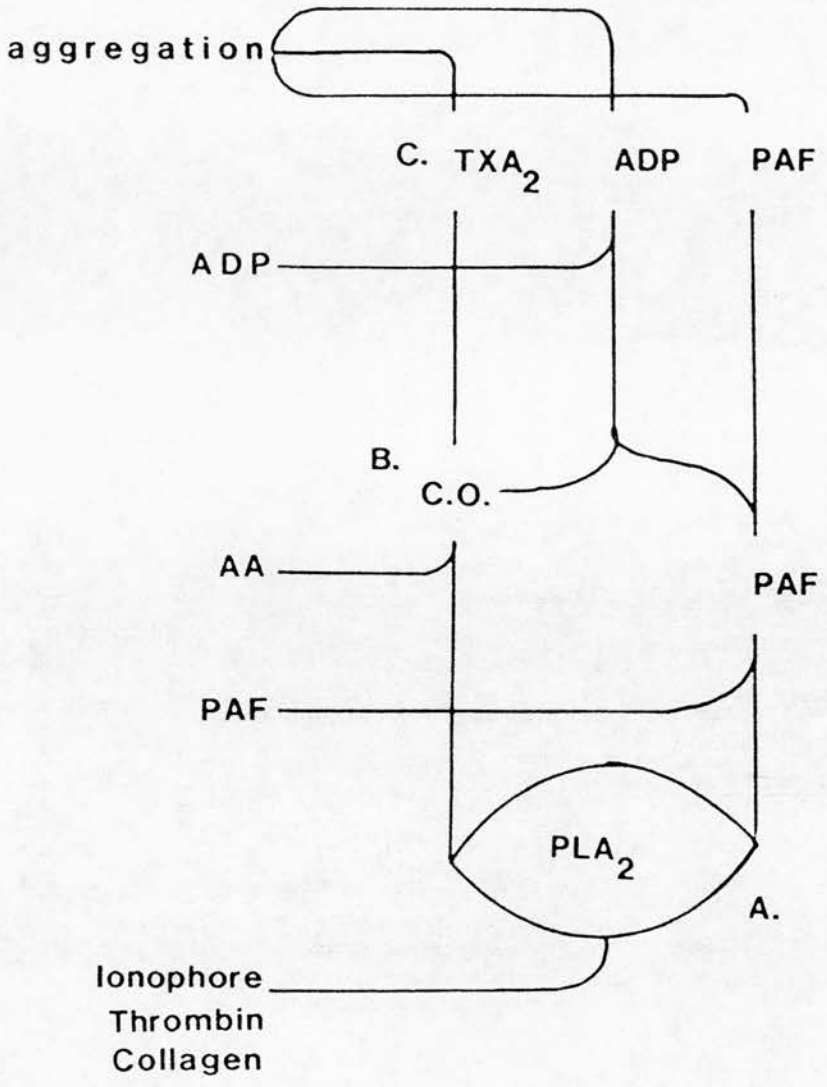
Agents which promote secondary wave aggregation, whether they do so via pathway (A) or (B) can be distinguished in the presence of non-steroidal anti-inflammatory agents and/or ADP removing enzymes (creatinine phosphate or creatinine phosphokinase, CP/CPK). Indeed second phase aggregation induced by collagen (low concentrations) or ADP is inhibited by aspirin and other NSAIDs, indicating that these agents are dependent upon prostaglandin endoperoxide/TXA_2 synthesis to augment aggregation either by recruiting further platelets and/or directly triggering the release reaction. In contrast, the formation of irreversible secondary aggregates by agonists acting via pathway (B) are not blocked by NSAIDs. Such agents include thrombin, PAF and TXA_2 which can act directly on the platelet granules to trigger the release reaction (Kaplan et al, 1979). It should be noted that aggregation induced by high concentrations of collagen can overcome NSAID inhibition, possibly by promoting secondary aggregation via a direct effect on the dense granules. Although such a direct effect is apparent at high concentrations it is not likely to be of any importance at low collagen concentrations since NSAIDs completely abolish second phase aggregation in this instance (Packham et al, 1977B).

A third pathway of aggregation has been postulated which is independent of both TXA_2 and ADP. In the presence of NSAIDs and CP/CPK, thrombin induced aggregation is only partially inhibited, indicating the involvement of another mechanism (Kinlough-Rathbone et al, 1977; Packham, 1977).

It has been suggested that a phospholipid, 1-O-alkyl-2-acetyl-

glyceryl-3-phosphorylcholine (platelet activating factor, PAF) may be the mediator of this third pathway (Vargaftig et al, 1981). PAF induced aggregation does not require ADP secretion or TXA_2 synthesis (Cazenave et al, 1979). Experiments were carried out whereby radioactive acetate was incorporated into platelet membrane phospholipids and the demonstration that radioactive acetate was present in PAF as a consequence of calcium ionophore stimulation would suggest that PAF may play a role as an intracellular mediator of aggregation (Chap et al, 1981). Furthermore, platelets desensitized to PAF lose their ability to respond to thrombin.

The mode of PAF-acether release from platelet membranes is not fully understood. The calcium ionophore A23187 is the most active releasing agent (Chignard et al, 1977b). Collagen and thrombin release enough PAF to account for aggregation, whilst ADP, AA and PAF are without any effect on PAF release (Vargaftig 1977b, Chignard et al, 1980). Since all agents which induce PAF-acether formation also trigger the activation of platelet phospholipase A_2 (Bills et al, 1976) it has been proposed that this enzyme is implicated in PAF acether formation by platelets (Cazenave et al, 1979). The finding that inhibitors of platelet PLA_2 activation, namely dibutyryl AMP (Lapetina et al, 1977) and bromophenacylbromide (Vargaftig et al, 1980a) reduce or suppress PAF-acether formation in platelets stimulated with the calcium ionophore supports the involvement of PLA_2 in PAF-acether release. A role for PAF in the third pathway of aggregation has been schematically represented together with the AA and ADP pathways by Vargaftig in Fig.1.2.



Vargaftig et al, 1981

Figure 1.2 Schematic representation of three pathways of platelet activation. Inhibition is observed at: (A) by phospholipase inhibitors, (B) by cyclo-oxygenase inhibitors and (C) by prostaglandin endoperoxide/thromboxane A_2 receptor antagonists.

Evidence has been put forward, against such a role for PAF as mediator of the third pathway of aggregation (Kloprogge et al, 1983) based on the findings that NSAIDs, acetylsalicylic acid and CP/CPK completely inhibited PAF induced aggregation and secretion. It is possible that PAF-acether plays only a minor role, if any at all in platelet activation induced via a third pathway. Indeed it has been demonstrated that PAF-induced aggregation is accompanied by the release reaction and TXA_2 production. (Miller et al, 1982; Cargill et al, 1983; Lapetina et al, 1983).

In addition to the 3 mechanisms described above which require the presence of agonists, platelets can also aggregate as a result of close cell-cell contact, whereby activation of the AA pathway with the subsequent formation of TXA_2 can trigger the release reaction, which consequently promotes secondary aggregation. This type of aggregation is blocked by NSAIDs and in this situation the release reaction is secondary to close platelet contact and aggregation (Massini and Zuscher, 1971).

A number of situations have been reported in which release and secretion may occur without any aggregation being observed. For example, thrombasthenic platelets change shape and release their granule contents in response to thrombin but do not actually aggregate (Caen et al, 1966; Zucker et al, 1966). Another example is the observation that platelets can undergo the release reaction when aggregation is not apparent in the presence of calcium chelators. It would appear that although platelet contact associated with aggregation can induce TXA_2 synthesis and hence

trigger the release reaction, situations do exist whereby release and secretion can occur independently of aggregation.

Irrespective of the precise mechanism underlying the platelet response, whether it is shape change, primary or secondary wave aggregation, activation of platelets is initiated by agonist interaction at the surface membrane usually at discrete receptor sites. Surface receptors have been identified for ADP (Nachman and Ferris, 1974), thrombin (Detwiler and Feinman, 1973; Tollefsen and Majerus, 1976), adrenaline (Byddeman and Johnsen, 1969), 5HT (Michal, 1969) and certain prostaglandins and TXA_2 (Le Breton et al, 1979; Fitzpatrick et al, 1978; Nicolaou et al, 1979). 5HT and adrenaline, in addition to their action at discrete receptor sites, can be actively taken up into platelets, a phenomenon not associated with aggregation.

Most of the compounds mentioned above induce only aggregation. Metabolites of AA can either induce or inhibit platelet activation. The prostaglandin endoperoxides, PGG/PGH_2 and TXA_2 fall into the former category, acting at a TXA_2/endoperoxide receptor. There is a good deal of controversy as to the true biological activities of the endoperoxides. For example, it has been reported that thromboxane synthetase inhibitors virtually abolish AA aggregatory activity (Fitzpatrick and Gorman, 1977; Blackwell et al, 1978) and that the concentrations of endoperoxides detected in platelets following AA induced aggregation are 100 times lower than would be required for aggregation. In contrast, it has been demonstrated that the addition of exogenous PGG_2 to platelets 'in vitro' induces very rapid

aggregation and release (Claesson and Malmsten, 1977). The fact that exogenous PGH_2 is degraded in PRP predominantly to PGD_2 and PGE_2 with less than 1% conversion to TXA_2 (Smith et al, 1975) may possibly explain why low levels of endoperoxides are detected in some studies and why thromboxane synthetase inhibitors may block aggregation, since the direct action of PGG_2 or PGH_2 may be masked by their rapid transformation to PGD_2 a potent inhibitor of aggregation (Oelz et al, 1977). Other workers have suggested that AA conversion to TXA_2 is not essential for aggregation (Needleman et al, 1976, 1977; Flower and Cardinal, 1979) supporting the notion that endoperoxide themselves are capable of inducing aggregation.

It is generally assumed that the endoperoxides and TXA_2 act on a common receptor; the greater potency of TXA_2 predisposes one to designate the receptor a "thromboxane receptor". From a chemical viewpoint the majority of the TXA_2 mimetics studied are in fact endoperoxide-like in structure (Corey et al, 1976; Bundy, 1976). Furthermore, receptor antagonists are capable of blocking the aggregatory effects of both PGH_2 and TXA_2 suggesting that these antagonists are acting on a common receptor.

In spite of these findings, it has also been postulated that TXA_2 activates platelets by acting as a physiological ionophore transporting calcium directly from the dense tubular system of platelets to the cytoplasm (Gerrard et al, 1977B, 1978B). However more recently workers have disputed this role of TXA_2, and their argument against it was based upon studies using stable thromboxane antagonists. In the presence of these antagonists, calcium release

from intracellular stores in the intact platelet and from isolated vesicular membranes was inhibited, supporting a receptor mediated effect of thromboxane (Rybicki et al, 1983; Pollock et al, 1984). Furthermore, Carey and colleagues (1985) when studying the calcium sequestration nature of intracellular membranes, found that of the three compounds, A23187, 11,9-emPGH_2 and PGH_2, only the calcium ionophore A23187 had the ability to release sequestered calcium.

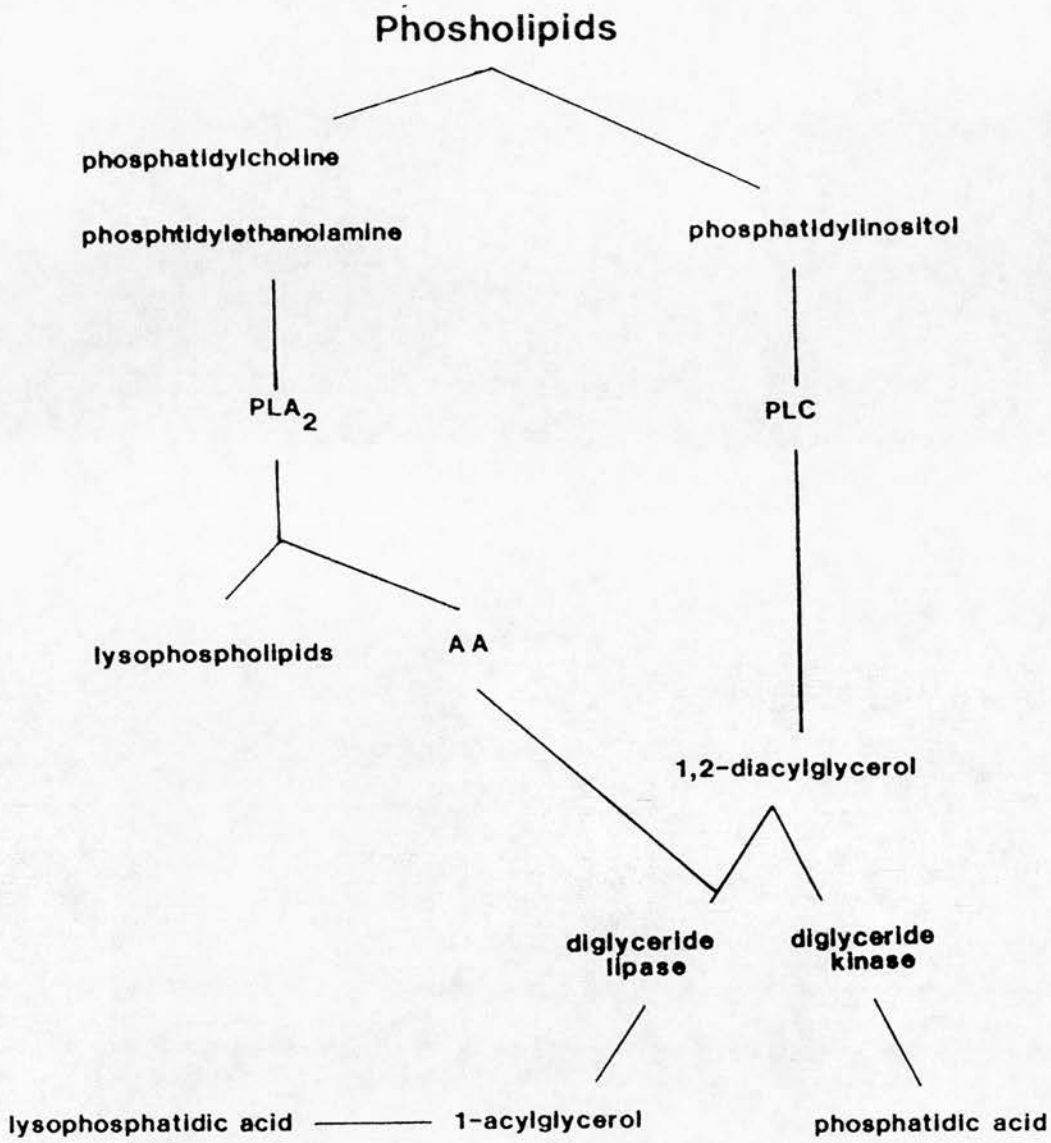
Platelets also contain receptor systems responsive to prostaglandins E_1, I_2 and D_2. Occupation of these receptors leads to an activation of adenylate cyclase with subsequent inhibition of platelet function due to elevations in cyclic AMP. Two distinct receptors for PGD_2 and PGI_2 have been identified (McIntyre and Gordon, 1977; Miller and Gorman, 1979) with PGE_1 acting on PGI_2 receptors.

Recently, PGE_1 has been reported to inhibit platelet aggregation independent of cyclic AMP involvement (Sinha and Colman, 1985). A macromolecular plasma protein is thought to be involved which exerts an inhibitory effect by altering a factor XA component. As yet, the precise mechanism underlying this inhibition remains to be elucidated.

Essentially, platelet responses are evoked by the interaction of agonists at specific receptors and it is appreciated that such responses are a sequelae of controlled intracellular reactions mediated by second messengers.

Phospholipase A_2, a membrane bound enzyme is stimulated by many agonists, and activation of this enzyme releases AA and possibly PAF acether from membrane phospholipids which initiates intracellular platelet mechanisms including Ca^{2+} fluxes and, cyclic AMP changes.

Another pathway for the release of AA from platelet phospholipids independent of PLA_2 activation has also been described (Lapetina and Cuatrecacas 1979, Bell and Majerus 1980, Mauco et al, 1978) and involves the activation of a phosphatidyl inositol phosphodiesterase (phospholipase C, PLC), stimulation of which results in the formation of diglycerides and polyphosphoinositides from phosphoinositol. The presence of this enzyme in platelets was first indicated from the presence of phosphatidic acid in platelets without any evident AA release (Lapetina and Cuatrecasas 1979). Phosphatidic acid is itself a potential trigger for aggregation. It releases calcium from platelet membrane fractions and therefore acts in many respects as a calcium ionophore (Gerrard et al, 1978). It cannot however cross platelet membranes and so if added exogenously to platelets, it does not possess the ability to trigger aggregation. A schematic representation of these 2 pathways is illustrated in Fig.1.3. Hydrolysis of phosphoinositides by PLC results in the transient accumulation of 1,2-diacylglycerol within the platelet. One of its function is to trigger the release of AA from membrane phospholipids (Rittenhouse-Simmons, 1979) as well as to activate protein-kinase C (Nishizuka, 1983). In addition diacylglycerides have been shown to induce aggregation without an apparent elevation in intracellular calcium above basal levels. Since endogenously formed diacylglycerol is present for a transient period only, it may



Hallam and Rink, 1984

Figure 1.3 Schematic representation of two enzymatic pathways triggering arachidonic acid release from membrane phospholipids.

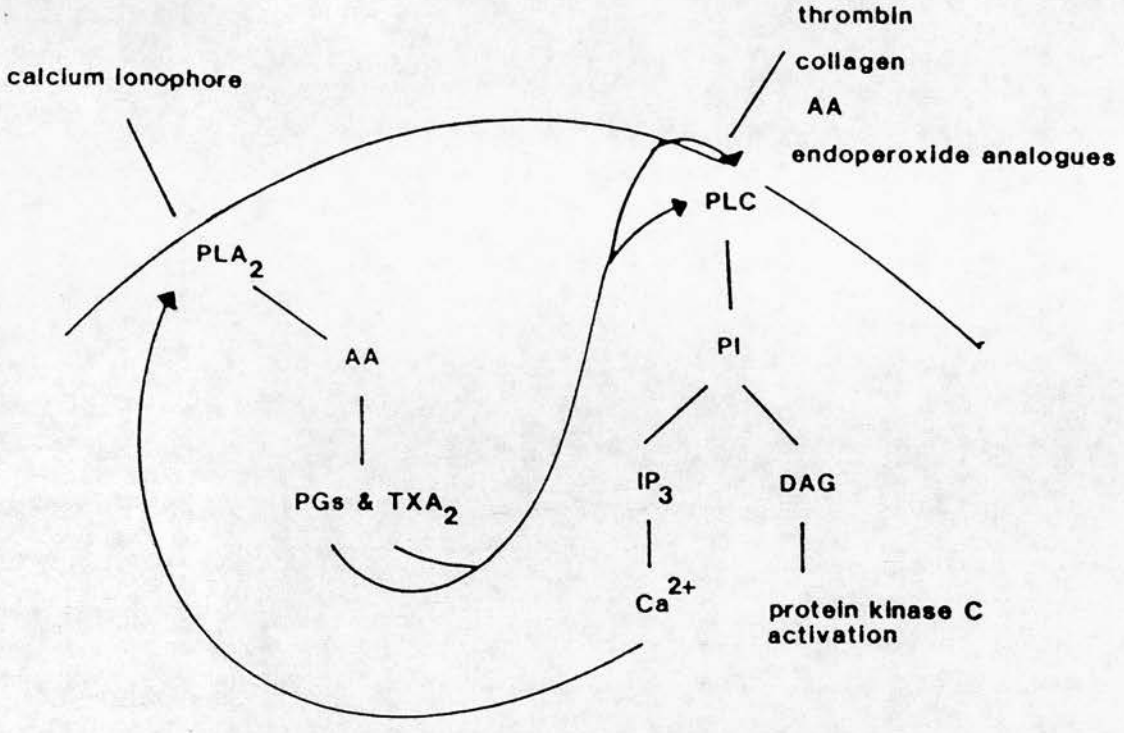
not exert any appreciable aggregatory effect alone under physiological conditions. However, responses of diacylglycerol resembling those of natural agonists (ADP, thrombin, etc.) can be evoked by the combined action of diacylglycerol and a modest elevation in intracellular calcium, whereby independently they would be inadequate to produce secretion and aggregation (Rink and Hallam, 1984). Another molecule generated from phosphoinositol is 1,4,5-inositol-triphosphate (IP_3) and it has been postulated to act as an intracellular messenger triggering calcium fluxes within platelets.

Stimulation of platelets by thrombin (Lapetina and Cuatrecacas, 1979), collagen (Nishizuka 1983; Seiss, 1983a), PAF (Lapetina and Siegel, 1983) and AA (Seiss et al, 1983b) is associated with rapid changes in membrane inositol phospholipids through activation of both PLC and PLA_2. Stimulation of PLC has been reported to occur before PLA_2 (Lapetina and Seiss, 1983b). PLC products may act as second messengers which act on the PLA_2 enzyme (Lapetina, 1983b). In human platelets stimulated with endoperoxide analogues (U44069 and U46619) a rapid formation of 1,2-diacylglycrol and phosphatidic acid has been reported (Pollock et al, 1984), a process which was found to be insensitive to NSAIDs. Thrombin too was found to activate PLC with the resultant formation of IP_3 in the presence of aspirin (Rittenhouse, 1985). On the other hand, the calcium ionophore did not activate PLC in the presence of aspirin, apyrase or CP/CPK, and it has been suggested that PLC is not actually activated by changes in calcium ions but rather by the presence of agonists acting through receptors (Rittenhouse, 1985). The calcium ionophore possibily activates PLC enzymes indirectly by stimulating PLA_2 (which is

sensitive to calcium fluxes). The latter releases AA and it is the prostaglandins and TXA_2 which induce PLC to generate polyphosphoinositides, and not the calcium levels which are elevated by the ionophore. These pathways are illustrated in Fig.1.4.

Calcium therefore, is an important second messenger involved in stimulus-induced platelets aggregation, secretion and generation of potent arachidonate metabolites. On the other hand cyclic AMP generated in platelets by stimulation of the adenylate cyclase enzyme, acts to prevent nearly all manifestations of platelet activation. Cyclic AMP may act in part by increasing intracellular calcium sequestration within the platelet (Owen et al, 1980) with a consequent effect on protein phosphorylation due to a restriction on the amount of cytoplasmic calcium available to these enzymes (Kawahara et al, 1980). Feinstein et al (1983) demonstrated that cytoplasmic free calcium levels are regulated by cyclic AMP dependent reactions in intact platelets. In their experiments they not only demonstrate that elevations in cyclic AMP supress a rise in cytoplasmic calcium induced by thrombin, but also that cyclic AMP can lower cytosolic calcium levels following stimulation by an agonist.

The precise mechanisms underlying the action of cyclic AMP on cytoplasmic calcium levels are not fully understood, but it has been suggested that cyclic AMP may inhibit a crucial initial biochemical reaction necessary for intracellular release of calcium or its influx from the surrounding media (Feinstein et al, 1983). An involvement of cyclic AMP on the stimulation of calcium ion transport and/or calcium binding has also been suggested. Inhibitory prostaglandins



PLA_2/C,	Phospholipase A_2/C enzymes
AA,	Arachidonic acid
PI	Phosphatidylinositol
IP_3	Inositol-1,4,5-triphosphate
DAG	Diacylglycerol

Figure 1.4 Interaction of enzymatic pathways involved in arachidonic acid release from platelet phosphilids.

and Forskolin (Insel et al, 1982) can stimulate processes that rapidly remove calcium from the cytoplasm induced by prior treatment with thrombin. Furthermore the lowering of cytoplasmic calcium by cyclic AMP stimulators closely corresponds both in the time course and dose-response to their ability to reverse thrombin induced aggregation, protein phosphorylation and cytoskeleton assembly (Feinstein et al, 1983B). The involvement of a cyclic AMP dependent protein-kinase on the stimulation of calcium ion reuptake and the phosphorylation of proteins participating in the active pumping of calcium ions from the cytoplasm to intracellular stores has been postulated (Haslam et al, 1978; 1979; Kaser-Glanzmann et al, 1977). Studies have also suggested that increases in cyclic AMP may depress phospholipase C activity in intact platelets and this effect will be of relevance to cAMP induced inhibition of cytosolic calcium fluctuations if phosphoinositide breakdown is functionally related to the mobilization of intracellular calcium (Rittenhouse, 1979). In this situation cyclic AMP would be modulating the response of calcium indirectly by exerting control at the level of the phospholipase C enzyme and so presumably regulating the level of diacylglycerol present in the system.

In addition to stimulating active sequestration of calcium, and the inhibition of the phospholipase C enzyme, several groups have reported that an elevation in cyclic AMP inhibits both arachidonate liberation from phospholipids (Gerrard et al, 1977; Lapetina et al, 1977) and possibly the cyclo-oxygenase enzyme (Malmsten et al, 1976).

The ability of cyclic AMP therefore to lower cytosolic calcium,

highlights an important role for cyclic AMP; as a modulator of (A) the initiation of platelet activation by aggregating agents and (b) in reversal of the activation process following stimulation.

It is apparent from this chapter that the platelet is a complex entity whose functional integrity is important in the maintenance of haemostasis. The platelet functions as a result of interactions of many biochemical processes occurring at both the surface membrane and the intra-cellular organelles. Only from pharmacological manipulations of these events has many of the underlying mechanisms been elucidated.

The work in this thesis has been concerned with only a few aspects of platelet function and can be divided into 3 sections. The first investigates the nature of AA induced aggregation in rat platelets since much conflicting data exists in this area. AA was found to evoke several responses in rat platelets and the aim was to elucidate the underlying mechanisms involved in each of the modes of action. The second section involves an investigation of the prostaglandin endoperoxide/thromboxane system in platelets from 3 species; human, rat and rabbit. Much of the published data on thromboxane-like action of platelets and smooth muscle appears to suggest that the platelet receptor is different from the corresponding smooth muscle receptor (Lefer et al, 1980), but whether these receptor differences reflect heterogeneity between tissues or perhaps between species is not clear. The overall aim of the work carried out in this section was therefore to compare the pharmacological characteristics of the thromboxane receptor of the

human platelet with the rat and the rabbit platelet in terms of their interaction with natural and synthetic prostanoids. The final section in this thesis concerns the function of platelets in two pathological conditions, namely diabetes mellitus and hypothyroidism. Diabetes is a disease which is unfortunately often associated with many other complications especially of the microvasculature. Retinopathy is one such complication and the effect of platelet disfunction as a possible cause of this condition was investigated by comparing platelet sensitivity in non-retinopathic diabetics to those with different degrees of retinopathy. In addition, platelet function and sensitivity was studied in 'uncontrolled' diabetics whose glycaemic control was improved over a six month period. Finally, hypothyroidism, like diabetes, is associated with other problems, including menorrhagia, increased incidence of arteriosclerosis and easy bruising. The study in this section was carried out on patients over a six month period who were undergoing hormone replacement therapy and their platelet reactivity was monitored over this period to see whether treatment improved or changed platelet function.

CHAPTER 2

Materials and Methods

This chapter describes the general procedures underlying the methods utilized in this thesis and the materials used.

Preparation of Platelet Suspensions and Measurement of Platelet Aggregation

Platelet preparation procedure

Blood was withdrawn from human subjects, rabbits and rats in the studies carried out in this thesis.

Human blood was withdrawn from the antecubital vein of volunteers, who had not previously taken any form of NSAIDs (for up to 7 days) and added to acid-citrate dextrose (ACD) anticoagulant (10mls/50mls blood) contained in plastic centrifuge tubes.

Rabbits were anaesthetized with Sagatal (30mg/kg body weight) (pentobarbitone sodium B.P., May & Baker Ltd., U.K.) injected into the marginal ear vein. Blood was withdrawn from both common carotid arteries through heparinized cannulae (100u/ml) (heparin - Boots Company Ltd., U.K.) and collected directly into plastic centrifuge tubes containing ACD (10mls/50mls blood).

Rat blood was withdrawn via the abdominal aorta of etherized rats, (diethylether, Rathburn Chemicals Ltd., U.K.) through heparinized cannulae (100u/ml) into ACD (1ml/10mls blood).

The blood from either human donors, rats or rabbits once

collected was centrifuged at 200 x g for 20 minutes allowing the red cells and other components to separate from the plasma. The upper platelet-rich plasma layer was then carefully drawn off into another plastic tube. At this stage the platelet-rich plasma layer was used directly or treated further to afford a washed platelet suspension. When platelet-rich plasma was used directly, it was left to stand at room temperature for 20-30 mins. (in the case of the human or rabbit) or 60 mins (in the case of rat plasma) allowing the platelets to restabilize following the centrifugation procedures.

Washed platelet suspensions

The conditions for preparing washed platelets in these experiments are not as rigorous as those used by other groups (Mustard et al 1972; Vargas et al, 1982) where several washing centrifugation steps are carried out. As a result, the washed platelet suspensions are not totally free of plasma factors including fibrinogen.

In order to prevent clumping of platelets during the second centrifugation step, prostacyclin (PGI_2) was added to the platelet-rich plasma prior to centrifugation (final concentration 10ng/ml). The platelet-rich plasma was then centrifuged at a minimum speed of 600 x g for a further 20 minutes allowing a platelet pellet to form. The platelet poor plasma was then carefully poured off and the platelet pellet was resuspended in calcium-free Krebs (3mls/ml PRP). The washed platelet suspension was then left for 1 hour before use, allowing time for the PGI_2 to decay and the platelets to restabilize.

During this 1 hour period and throughout the experiment, the platelet suspension and the Krebs' solution were bubbled with 95% O_2/5% CO_2. This procedure prevents the pH of the platelets and the Krebs' solution from increasing, which normally occurs with time if platelets are not aeriated.

Platelet aggregation

Platelet aggregation was measured by the turbidometric method of Born (1962). Each cuvette contained:-

	Vol. (ml)
platelet suspension (PRP or washed)	0.5
Krebs solution	0.3
Saline (0.9%)	0 - 0.2
Vehicle	0 - 0.1
Total volume	1 ml

All cuvettes and stirrer bars (teflon coated) were siliconized with 2% dichlorodimethylsilane in toluene and washed twice with methanol before use. A Bryson aggregometer (H. Upchurch & Co., Ltd.) was used and the changes in light transmission of the platelet suspension were recorded on a potentiometric recorder (Servoscribe IS).

The cuvette, containing the platelet suspension was placed in the aggregometer and incubated for 2 minutes at 37°C with constant stirring (1100 rev/min). When studying the activity of antagonists or inhibitors, these were added to the platelet suspension prior to the 2 min incubation period. Agonists were then added and incubated

for a further 2 mins.

Measurement of the platelet release reaction

Platelets are known to release the contents of their granular storage vesicles when activated by aggregating agents. A method is described below which allows a quantitative determination of the amounts released from these granules and is based upon the principle that platelets can actively take up and store 5HT and adrenaline into their dense bodies.

Platelet rich plasma from blood was pre-incubated with radiolabelled 5HT (5-hydroxy-(side chain)2-[^{14}C]-tryptamine, specific activity 143nCi/µg, final concentration of 1µg/ml^{-1}) for 30 minutes at 37^{o}C. The [^{14}C]-5HT is actively taken up by the platelet into the dense granular bodies during this pre-incubation period.

Following the incubation period, platelet rich plasma was used in aggregation studies as previously described. In order to estimate [^{14}C]-5HT release, at the end of the 4 minute period, 200µl samples (X2) were withdrawn from the cuvette and added to 0.8ml ice-cold ethylenediaminetetra-acetic acid (E.D.T.A. solution 0.4% w/v in 0.9% saline) in an Eppendorf tube. The quenched samples were centrifuged immediately at 15000 x g (20^{o}C for 30 seconds) in a bench Eppendorf centrifuge. Subsamples (0.5ml) of the cell free supernatant were then transferred into 10ml of scintillant (P.C.S. and toluene 2:1).

Radioactivity was counted in a Philips PW4540 liquid scintillation counter. Sample radioactivity was counted and expressed as counts per minute and the amount of radioactivity released per sample was calculated as follows;

Final concentration of [^{14}C]-5HT	1µg/ml
Specific Activity of [^{14}C]-5HT	143 nCi/µg
In 0.5ml PRP sample we have	71.5 nCi
Withdraw 200µl (1/5 total volume)	14.3 nCi
Withdraw further 500µl (1/2 of above vol.)	7.15 nCi

The amount of [^{14}C]-5HT releasable was then converted from disintegrations per minute (d.p.m.) to counts per minute (c.p.m.) using the following formula;

$$0.00715\mu Ci \times 2.2 \times 10^6 \text{ cpm} = 15730 \text{ dpm}$$

Correcting for counting losses, the maximal releasable [^{14}C]-5HT was calculated to be 11,450 dpm.

The amount of radioactivity released per sample was then expressed as a percentage of the maximal releasable radioactivity.

Estimation of platelet count

Reagents used: Solution A (100ml) 1g Sodium citrate
0.002g Mercuric chloride
0.2g Brilliant Cresyl Blue
Warmed to 45°C
Solution B (100ml) 20g Urea

Method

Estimation of the platelet number in a sample of plasma or washed platelet suspension was determined by the Cresyl Blue Dye method. Equal volumes of solutions A and B were mixed and filtered. An aliquot (50µl) of platelet rich plasma or washed platelets was added to 1ml of the dye mixture, and samples taken up by a glass capillary and used to fill both chambers of a haemocytometer. Sedimentation was allowed to proceed for 15 minutes in a petri-dish containing moist filter paper. An aliquot (10µl) of the dye-platelet mixture was placed in a counting chamber, marked with a grid. The number of platelets in 5 squares of the grid were counted and the mean value was multiplied by 10^4, giving an estimate of the number of platelets per µl of PRP or washed platelet suspension.

Mean number of platelets ($x10^8$) ml^{-1} of:

	PRP	Washed Platelet Suspensions
Human	85 ± 8	89 ± 5
Rabbit	86 ± 12	115 ± 15
Rat	107 ± 15	141 ± 18

Stock solutions of arachidonic acid (10mg/ml) were made up in ethanol and stored at -20°C. For the aggregation studies an arachidonate sodium salt (3mg/ml) was made up. A volume of 0.6ml of stock arachidonic acid (in ethanol) was added to 20µl of NaOH (1M). The ethanol was blown off under nitrogen and 0.9% NaCl (2ml) was added to give a 3mg/ml arachidonate solution. Subsequent working dilutions were made up in 0.9% NaCl.

Stock solutions of fatty acids were also made up in ethanol and stored at -20°C. Working dilutions were made up following the same procedure as for arachidonic acid.

All fatty acids were tested for the presence of impurities or any degradation products by thin layer chromatography (T.L.C.). Specific analysis of endoperoxide content was not carried out but the presence of any contaminants was not apparent from the T.L.C. analysis.

The aggregation solutions (ADP, collagen etc.) used in the longitudinal studies were standardised as far as possible. Fresh stock solutions (originally made up from the same batch) were used at the beginning of each study period (0, 3 and 6 mths) and fresh working dilutions were made up on the day of individual experiments.

Throughout this thesis the results are given as the mean of at least 6 experiments and aggregation recordings were carried out in duplicate.

Estimation of plasma-protein concentration in washed platelet suspensions

The residual plasma-protein concentration in washed platelet suspensions was estimated by ultra-violet spectroscopy as follows:-

(i) A sample of the washed platelet suspension (2.5ml) (platelet-free) was placed in a quartz cuvette and its U.V. extinction at 280nm recorded.

(ii) The P.R.P. was serially diluted until an extinction of similar intensity to that of washed platelet suspension was obtained.

(iii) Assuming that one U.V. extinction is equivalent to 1mg ml^{-1} of protein, one can estimate the protein concentration in PRP and washed platelet suspensions (Table 2.1).

Table 2.1 Plasma Protein Concentration (mg ml^{-1}) in PRP and washed platelet suspensions, (± s.e. mean of 3 samples)

	PRP	Washed platelet suspension
Human	51.5 ± 2.65	1.50 ± 0.15
Rat	50.4 ± 2.31	1.26 ± 0.13
Rabbit	40.71 ± 7.50	1.30 ± 0.06

Radioimmunoassay of Thromboxane B_2 (TXB_2)

The underlying principle of radioimmunoassay depends upon competitive binding, where labelled and unlabelled antigen compete

equally for antibody binding sites. A well designed antigen-antibody equilibrium reaction is not affected by radionucleotide labelling. The distribution of radioactive counts in the bound and free states is proportional to the relative concentration of unlabelled antigen. In general the purpose of a radioimmunoassay is to determine the concentration of unlabelled antigen in a biological sample.

A. Radioimmunoassay Procedures

A standard curve was set up whereby a series of tubes are prepared, each containing exactly the same amount of tracer (labelled antigen) and antibody reactants. Increasing known amounts of unlabelled antigen (standards) are added to successive tubes. The percentage of radioactivity (tracer) B/B_o to the antibody is inversely proportional to the amount of unlabelled antigen present in the tube.

The TXB_2 antibody used in experiments throughout this thesis was obtained commercially from The Pasteur Institute, France, in the freeze-dried form, and before use was reconstituted in phosphate buffer pH 7.5 (5.5 ml). Thromboxane B_2 levels are believed to reflect the levels of the active precursor thromboxane A_2 present from biosynthetic transformations of arachidonic acid. Radiolabelled TXB_2 (^3H-TXB_2) was obtained from Amersham PLC Ltd. UK (specific activity 6.66 TBq/mMol). The ^3H-TXB_2 was stored in methanol (20 µCi/ml) at -20°C and before use the methanol was evaporated off at 45°C under a stream of nitrogen and resuspended in

phosphate buffer (pH 7.5) to give a final concentration of 0.1 μCi/ml.

The following conditions were found suitable for TXB_2 radioimmunoassay;

Standard/unknown sample in buffer	0.50ml
TXB_2 antibody	0.05ml
Tracer - [^{3}H] TXB_2	0.05ml
Donkey - anti-rabbit serum (DARS)	0.05ml
Normal Rabbit serum (NRS)	0.05ml
Total volume	0.7 ml

In addition to the standards, a set of reference tubes were prepared; counting standards containing only ^{3}H-TXB_2 give a measure of the total radio-active counts per tube. Zero standards - no unlabelled antigen present, give an estimation of the maximum possible binding of the tracer to the antibody. Non-specific binding (NSB) tubes, containing excess unlabelled antigen, to saturate the antibody binding sites give an indication of the degree of binding of the tracer to other components of the assay, e.g. tube walls, or other potential binding sites. NSB usually accounts for 10% or less of the bound.

A series of standard solutions (5 pg ml^{-1} to 1000 pg ml^{-1}) of unlabelled TXB_2 was made up in phosphate buffer (pH 7.5) diluent from stock solution of TXB_2 (1 μg ml^{-1}) in methanol. TXB_2 standards (0.5 ml) were added to the tubes in duplicate as detailed in Fig.2.1.

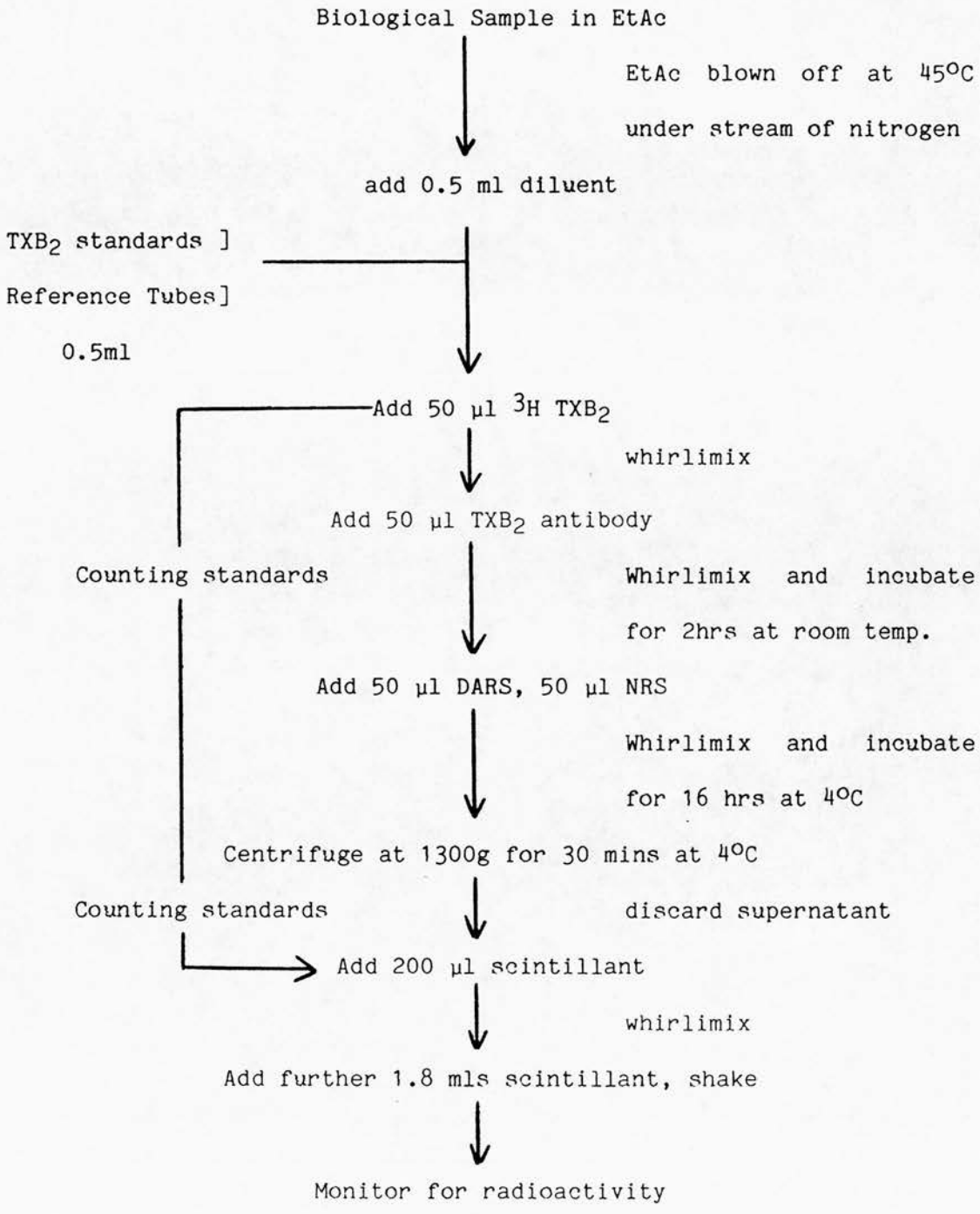
Fig.2.1 Concentration, volume and the final concentration of TXB_2 standards used in setting up the TXB_2 Standard Curve

Conc. of TXB_2 standard $pgml^{-1}$	Volume tube $^{-1}$ ml	Final TXB_2 conc.tube^{-1} pg/ml
600	0.5	300
300	0.5	150
160	0.5	80
80	0.5	40
40	0.5	20
20	0.5	10
10	0.5	5
5	0.5	2.5

Counting standards (0.05 ml ^3H TXB_2 only), Zero standards (0.5 ml diluent), and NSBs (0.5ml of 1 µg ml^{-1} TXB_2) were set up in quadruplicate.

Assay tubes containing the biological samples (with unknown amount of antigen) were prepared (in duplicate) and set up in the same manner as for the standards. It is essential that the standards and samples are subjected to the same experimental conditions. Fig.2.2 illustrates the procedural steps for standards, reference and sample tubes.

Fig.2.2 Methodology for TXB_2 standard curve and sample assay



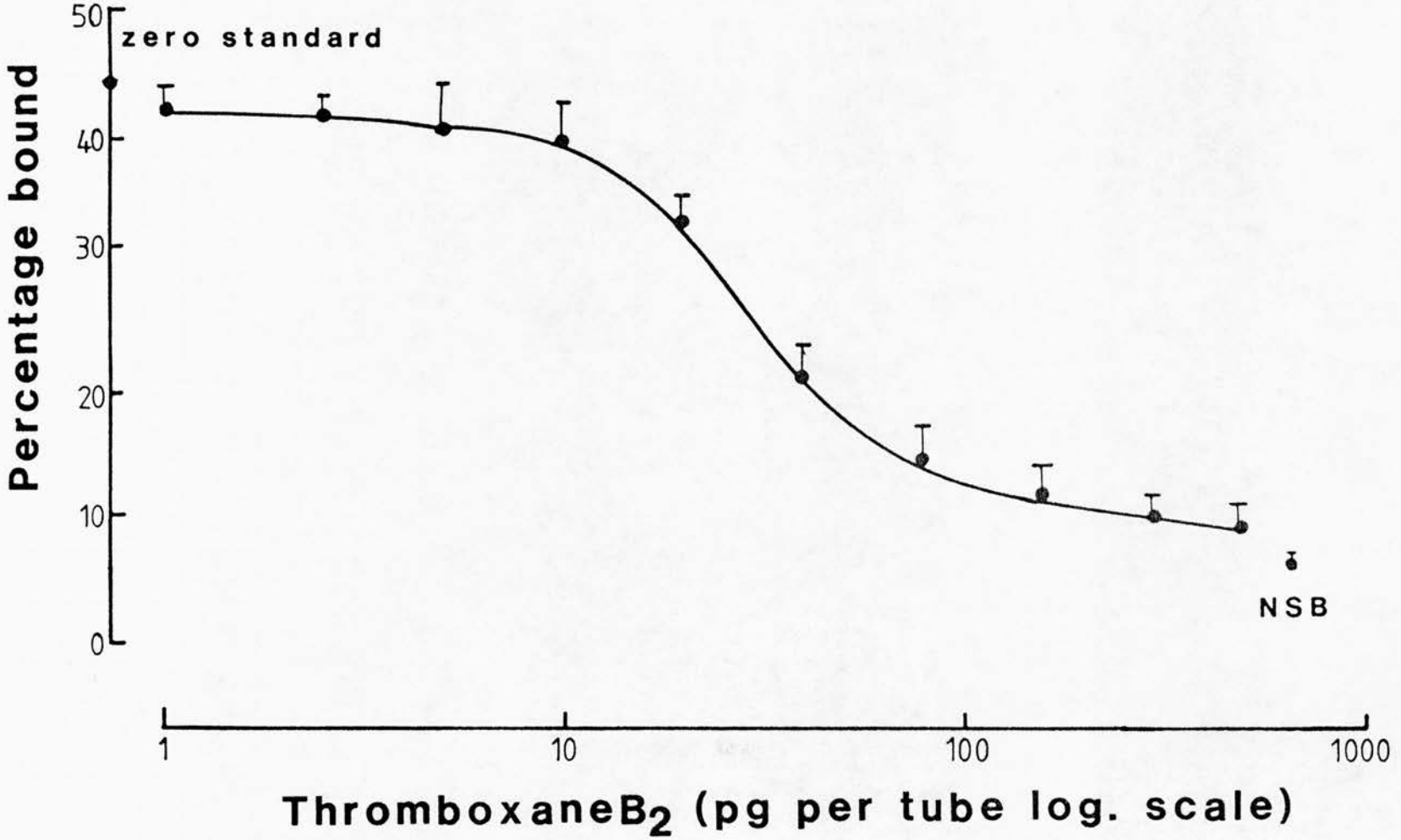
Separation of the bound tracer from the free is based upon the double antibody separation technique in which the bound antigen/antibody complex binds to donkey-anti-rabbit serum (DARS). Normal rabbit serum (NRS) is added to the system during this incubation period. The bound tracer/TXB_2 antibody/DARS complex is separated from the free tracer by centrifugation during which the heavier precipitated fraction sediments to the tube bottom.

B. Counting and Data Analysis

The number of counts per vial were recorded using the Packard 4000 series scintillation counter. The standard curve is plotted as % tracer bound (vertical axis) against concentration of TXB_2 (horizontal axis) and a logistic curve formula matches a hyperbolic curve model with the best fit curve statistics. The average deviation between the computer curve and standard points is estimated and if this deviation is more than 5%, the curve is not accepted. If the gradient is less than 8%, then small changes in the amount of TXB_2 would produce changes in the % of the tracer bound, too small to be accurately measured. In addition, if the curve is too steep the working concentration range of the assay is decreased considerably. In these situations, the curves are not generally accepted.

Fig.2.3 illustrates the standard curve drawn up from 6 individual assays (mean ± s.e. mean). The limit of sensitivity of each assay was defined as the concentration of the unlabelled TXB_2 which produces a 10% decrease in the binding of the zero standards and from Figure 2.3 the limit of sensitivity was approximately 25 pg TXB_2.

Figure 2.3 Standard curve for TXB_2 radioimmunoassay. Results are mean ± s.e.m. of 6 consecutive assays.


50
zero standard
40
30
20
10
0
Percentage bound
NSB
1
10
100
1000
ThromboxaneB2 (pg per tube log. scale)

Inter-assay and Intra-assay coefficient of variation

The inter-assay and intra-assay coeffients of variation provide an estimate of the accuracy within an experiment and the reproducibility of individual experiments respectively.

The intra-assay coefficient of variation for the assay was calculated using the following formula:

$$\text{Intra-assay coefficient of variance} = \sum \frac{\text{Standard Deviation of assay duplicate}}{\text{mean}} \times 100$$

and for TXB_2 in an assay was calculated to be 4.6%.

The inter-assay co-efficient of variation (from 5 experiments) was determined for 80pg TXB_2 from the following formula;

$$\text{Inter-assay coefficient of variance} = \sum \frac{\text{Standard Deviation of standard from 5 expts.}}{\text{mean of standard}} \times 100$$

and for TXB_2 assays, the inter-assay co-efficient of variance was calculated to be 3.2%.

Both the intra and inter-assay coefficient of variance should fall within 0-6% for assay accuracy and reproducibility to be acceptable.

Cross-Reactivity

The cross-reactivity of the TXB_2 antibody with other prostaglandins had been predetermined by the Pasteur Institute, France (Table 2.2).

The percentage cross-reactivity of the antibody is determined by estimating the concentration of prostaglandin which produces a 50% inhibition of the binding of tracer and then applying the formula below;

% Cross reactivity =

$$\frac{\text{Conc. of } TXB_2 \text{ binding 50\% tracer}}{\text{Concentration of PG or metabolite binding 50\% tracer}} \times 100$$

Table 2.2 Cross-reactivity of rabbit TXB_2 antibody measured at B/B_o = 0.5 (equivalent to 50% inhibition of binding of tracer).

Prostaglandin	% cross-reactivity
PGA_2	0.10
PGB_2	0.10
PGD_2	0.20
PGE_2	0.10
$PGF_{2\alpha}$	0.10
TXB_2	100.0

In the radioimmunoassay of TXB_2 from arachidonate aggregation experiments (Chapter 3, Fig.3.6) any interference due to high concentrations of arachidonic acid (100μM) was not accounted for. It is possible that the high levels of TXB_2 measured with 500μM or more arachidonate could be due to some cross-reactivity of the arachidonate itself with the TXB_2 antibody. Pre-incubation of arachidonate samples with a cyclo-oxygenase inhibitor would possibly allow a quantitative estimation of the extent of interference in the radioimmunoassay of TXB_2.

Extraction and Preparation of Biological Samples

Thromboxane B_2 was extracted from either platelet-rich plasma or washed platelet suspensions, the method used is described below.

The PRP/washed platelet suspensions (1ml) were added to 10ml siliconized glass test tubes. Following the addition of 200µl collagen solution (2µg ml^{-1}) the tube contents were mixed and incubated at 37°C for 2 minutes. An aliquot of 2M HCl (200µl) was added at this stage to quench the reaction, the tubes whirlimixed and 10ml ethyl acetate was added. The tube contents were mixed thoroughly and centrifuged on a bench centrifuge for 5 minutes. The ethyl acetate layer was removed and stored at -20°C until assayed. Basal TXB_2 were set up as above but omitting the addition of collagen.

In aggregation experiments, the contents of the cuvette were directly added to 10ml siliconized test tubes containing 200µl HCl (2M) following aggregation, whirlimixed and 10mls ethyl acetate was added. The samples were treated as described above.

Before assaying for TXB_2, the ethyl acetate was evaporated under a stream of air at 45°C and the dried down samples were taken up in a known volume of ethyl acetate. Aliquots of the samples in ethyl acetate were dispensed into radio-immunoassay tubes and the ethyl acetate blown off at 45°C under a stream of air. A fixed volume of assay diluent (0.5ml) (phosphate buffer pH 7.5) was added to these tubes which were now ready to be assayed.

Recovery of tritiated TXB_2 from blood plasma samples

A standard aliquot of ^3H-TXB_2 in diluent was added to 1ml of PRP in siliconized test tubes (in quadruplicate) and the extraction procedure described above was carried out (omitting the addition of collagen). A similar aliquot of ^3H-TXB_2 in diluent was added directly to scintillant (3.5 mls) (in quadruplicate). These tubes give a reference of the total counts.

Following the extraction procedure the ethyl acetate taken off was evaporated to dryness and the residue was redissolved in 2mls ethyl acetate. The ethyl acetate was transferred to a scintillation vial and evaporated to dryness under air at 45°C. Scintillant (3.5 ml) was added to these tubes. Radioactivity was counted and the percentage recovery was calculated using the formula below;

$$\% \text{ Recovery} = \frac{\text{mean disintegrations per min. (d.p.m.) per vial}}{\text{mean d.p.m. of counting standards}} \times 100$$

The percentage recovery (mean ± s.e. mean, n = 4) was calculated to be 84.0 ± 3.2%. Since the method described for the extraction procedure gave a high percentage recovery of TXB_2, TXB_2 levels measured in radio-immunoassay experiments were not corrected for procedural loss.

Cyclic AMP Protein-binding Assay

This assay is based upon competition between unlabelled cAMP and a fixed quantity of ^3H labelled compound for binding to a protein

which has a high specificity and affinity for cAMP (Gilmamn, 1970). Separation of protein-bound cAMP from the unbound nucleotide is achieved by adsorption of the free nucleotide onto charcoal, followed by centrifugation step whereby the bound fraction remains in the supernatant whilst the charcoal forms a pellet.

Following centrifugation, an aliquot of the supernatant is removed for liquid scintillation counting; this gives an estimate of the bound ^{3}H cyclic AMP. The concentration of an unknown amount of unlabelled cyclic AMP in a sample is then determined from a standard curve.

The assay protocol is given in Table 2.3.

The assay buffer is 0.05 tris pH 7.5 containing 4 mM Na EDTA (EDTA is a phosphodiesterase inhibitor and prevents the breakdown of cAMP .(Eheung W.Y 1970)).

^{3}H-cAMP (8-^{3}H adenosine 3'5'-cyclic phosphate ammonium salt was obtained from Amersham Radiochemicals (Sp. Act. of 30 Ci/mmol)). This was diluted 2000-fold in the assay buffer so that 50µl is equivalent to 0.9 pmol cAMP (0.025 µCi). The adenosine 3',5'-cyclic phosphate standard was obtained as the free acid from Sigma.

The binding protein was diluted 10-fold with assay buffer containing 0.1% bovine serum albumen (Sigma) which was reported to promote cAMP binding by protein kinase. Tubes 1-3 determine the cpm bound in the absence of the binding protein and the mean value is

Table 2.3 Cyclic AMP assay protocol

Tube number & description	Volume (µl) of: buffer	standard	unknown	^{3}H-cAMP	Binding Protein
1/2/3 charcoal/blank	150	-	-	50	-
4/5/6 0 pmol.cAMP	50	-	-	50	100
7/8/9 0.6 "	-	50	-	50	100
10/11/12 1.25 "	-	50	-	50	100
13/14/15 2.5 "	-	50	-	50	100
16/17/18 5.0 "	-	50	-	50	100
19/20/21 10 "	-	50	-	50	100
22/23/24 100 "	-	50	-	50	100
				50	100
25/26 (unknowns)	-	-	50	50	100
27 etc. "	-	-	50	50	100

subtracted from the cpm counted for the remaining tubes, giving the true cpm bound to the binding protein. Tubes 4-24 determine the binding over the range of the standard curve, i.e. 0-100 pmols cAMP. Tubes 25 onwards are the sample tubes to be assayed; two 200 µl aliquots are counted.

The tubes are whirlimixed and left on ice for two hours. At least 15 minutes before the end of the incubation period, the charcoal adsorbent was prepared by mixing 520 mg GSX charcoal and 400 mg bovine serum albumen with 20ml distilled water. This suspension was stirred constantly on ice, and 100 µl of the charcoal adsorbent was added to 16 tubes at a time. After whirlimixing they were centrifuged for 2 minutes at 12000g in a refrigerated centrifuge. A 200µl sample was removed from each tube, added to 10ml scintillant fluid (I) and counted on a Philips PW4540 liquid scintillation analyser for 4 minutes. By carrying out the charcoal precipitation step on only 16 tubes at a time, it ensures that the charcoal was present for a maximum time of approx. 6 minutes. During this period, the charcoal will bind free cAMP but with longer time intervals the charcoal will strip and bind protein-bound cAMP.

This effect is minimised by carrying out this stage at 2-4^{o}C and so the charcoal is kept on ice throughout the experiment.

The displacement of ^{3}H-cAMP over the range of the standard curve is shown in Table 2.4

Table 2.4 Displacement of ^{3}H cAMP binding over the range of the standard curve (0 - 100 pmol cAMP).

pmol cAMP	CPM Bound	Standard Error
0	4798	± 303
0.60	3655	± 320
1.25	3111	± 301
2.50	2312	± 310
5.00	1267	± 150
10.00	1754	± 121
100.00	1099	± 25

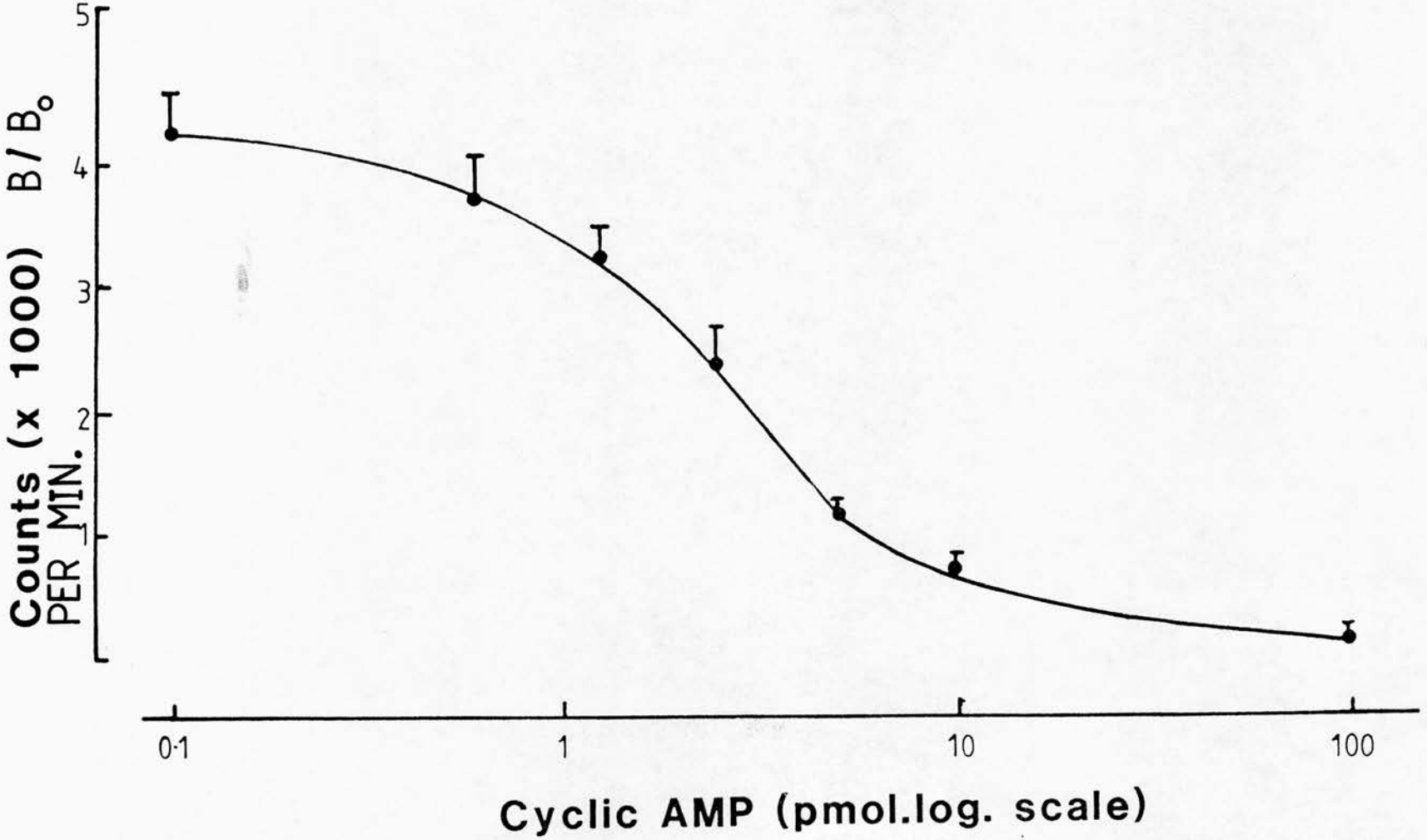
Fig.2.4 illustrates the cAMP standard curve over a concentration range of 0-100 pmol (each observation is the mean ± s.e. mean of 4 experiments).

The inter-assay and intra-assay co-efficient of variation

The inter-assay co-efficient of variation for 5.0 pmol cAMP was determined from the following formula;

$$\text{Inter-assay co-efficient of variation} = \sum \frac{\text{Standard deviation of 4 experiments}}{\text{Mean}} \times 100 = 11.76\%$$

Figure 2.4 Standard curve for cyclic AMP assay.
Results are mean ± s.e.m. of 4 consecutive assays.


Counts (x 1000) PER MIN.
B/B$_o$
5
4
3
2
1
0·1
1
10
100
Cyclic AMP (pmol.log. scale)

The intra-assay co-efficient of variation was determined from 4 individual assays, using the following formula;

$$\text{Intra-assay co-efficient of variation} = \sum \frac{\text{Standard deviation of triplicate}}{n} \times 100$$

where n = no. of assay triplicates, and for the cAMP assay was calculated to be 3.75%.

Extraction Procedure for cAMP

(i) PRP or washed platelet suspension (1ml) was added to a 10ml siliconized test tube and incubated for 30 secs at 37°C (basal camp levels). In order to measure elevation in cAMP levels, Iloprost, a stable prostacyclin mimetic was added to the PRP or washed platelet suspension (final concentration 0.03μM) before incubation and whirlimixed.

(ii) Samples (1ml) from aggregation experiments were added directly to 10ml siliconized test tubes.

Following procedures I and II the reaction in the test tubes was quenched by the addition of 2ml ethanol, the tubes were whirlimixed and left to stand for 5 minutes at room temperature. After centrifugation at maximum speed on a bench centrifuge, the supernatant was decanted into small glass assay tubes. The supernatants were evaporated to dryness at 55°C under stream of air and the residue was dissolved in 0.5ml assay buffer and centrifuged at 12000 xg for 30 minutes to remove insoluble material. Two 50μl

samples of the supernatant were assayed directly or if necessary the supernatant diluted in buffer.

Liquid Scintillation Counting

Radioactivity in samples was counted by adding aliquots to 10ml scintillant and counting for 4 or 10 mins using either the Philips PW4540 Liquid scintillation analyser or the Nuclear Chicago Liquid scintillation counter. Sample radioactivity was expressed in counts per minute (cpm) and these were converted to disintegrations per minute (dpm) by correcting for the amount of quenching in the sample.

Unless it is certain that all biological samples are chemically identical, except for the radioactive content, one cannot accurately compare cpm obtained from one set of experiments to another. Thus an appropriate means of standardisation can be carried out by correcting for quenching where the efficiency of counting for each sample is determined. Efficiency is defined as the ratio of the observed cpm to the true dpm.

To determine the efficiency of counting, scintillation counter machine constants are first of all determined by constructing a quench curve using a calibrated tritiated standard, ^{3}H-hexadecane, to provide a known number of dpm. The cpm were determined at different levels of quenching, using chloroform ($CHCl_3$) as the quenching agent (Table 2.5).

Throughout this thesis, both the Philips PW4540 and the Nuclear

Table 2.5 Quench Curve Protocol

Vial No.	µl Chloroform	DPM added	Scintillant I or II
1/2/3	-	-	10 mls
4/5/6	-	42250	"
7/8/9	5	"	"
10/11/12	10	"	"
13/14/15	20	"	"
16/17/18	50	"	"
19/20/21	100	"	"
22/23/24	200	"	"
25/26/27	300	"	"
28/29/30	400	"	"
31/32/33	500	"	"
34/35/36	600	"	"
37/38/39	1000	"	"

Chicago Liquid Scintillation counters were used as well as two types of scintillant (I and II - see materials). Machine constants for both counters and the two scintillants were evaluated from the quench curves (Fig.2.5) and listed in Table 2.6.

The quench curve is plotted by measuring effiency of counting, CPM/DPM (y axis) against ratio (x-axis), either the sample channel's ratio (S.C.R.) or external standard ratio (E.S.R.) can be used. The quench curve is described by the equation;

$Y = K_o + K_1X + K_2X^2$ where K_o, K_1 and K_2 are constants. Typical quench curves and the values of K_o, K_1 and K_2 are given in Fig.2.5.

These constants are used to determine the efficiency of counting in each sample so that DPM can be calculated accordingly.

Isolated Preparations

Rabbit Aorta

Thoracic aorta from male rabbits (2-3 kg) were removed immediately after they had been killed. Any connective tissue was carefully removed and spiral strips (3mm wide) were cut and suspended in 8ml organ baths containing Krebs' solution at 37°C and gassed with 95% O_2/5% CO_2 (B.O.C.). Tension changes were recorded with a Grass FT03 force displacement transducer linked to a Grass Polygraph. The resting tension for the rabbit aorta was set at 2g and the recorder full scale reflection at 4g.

Figure 2.5 Typical quench curves for tritium using chloroform for the quenching agent on a Phillips PW4540 counter using (A) ethoxyethanol/PPO/toluene and (B) PCS scintillants.

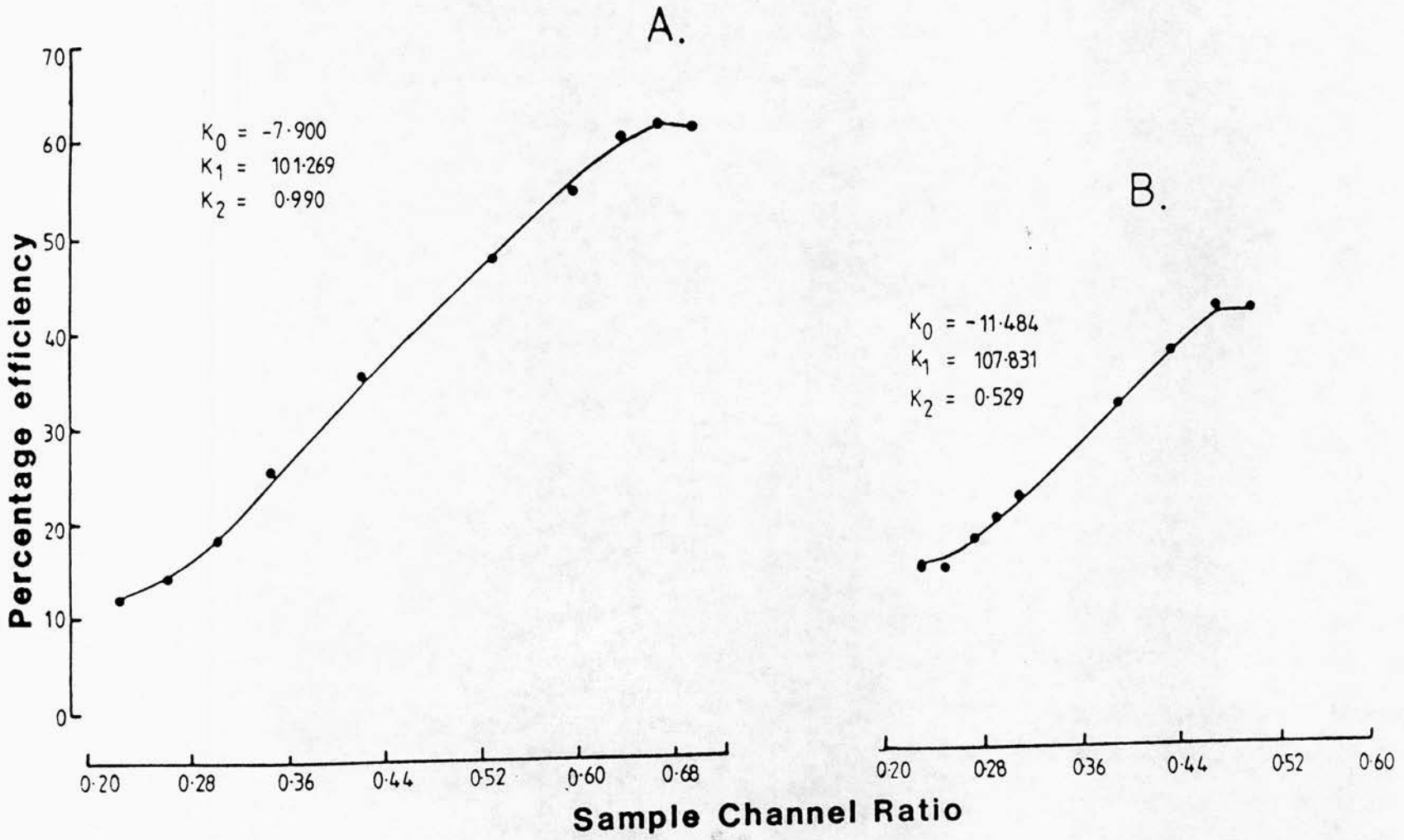

A.
K_0 = -7·900
K_1 = 101·269
K_2 = 0·990
B.
K_0 = -11·484
K_1 = 107·831
K_2 = 0·529
Percentage efficiency
0
10
20
30
40
50
60
70
0·20
0·28
0·36
0·44
0·52
0·60
0·68
Sample Channel Ratio

Table 2.6 Quench curve constants determined for the two types of scintillant used, in the Philips PW4540 and Nuclear Chicago liquid scintillation counters.

Scintillant	Constants	Liquid Scintillation Counter Philips PW4540	Nuclear Chicago
A. I (Ethoxyethanol/ (PPO/toluene)	K_o	- 11.484	91.979
	K_1	107.832	- 95.287
	K_2	0.529	1.125
B. II (PCS scintillant)			
	K_o	- 7.913	83.918
	K_1	101.269	- 130.612
	K_2	0.994 (S.C.R.)	0.649 (E.S.R.)

Each preparation was allowed 2 hours to relax to a stable resting tension during continual upward displacement of the bathing fluid (one nominal bath clearance per minute).

Guinea-pig trachea

Trachea from female guinea-pigs (600-950g) were removed immediately after the guinea-pigs had been killed. Spiral strips were (3-4mm wide, 8-10mm long) cut and suspended in 8ml organ baths containing Krebs' solution. In addition the bathing fluid contained atropine sulphate (2×10^{-8}M) and indomethacin (10^{-6}M). Tension changes were recorded witha Grass FT03 force displacement transducer linked to a Grass Polygraph as for the rabbit aorta. The resting tension for the guinea-pig trachea was set at 0.5g and full scale deflection at 2g. Again each preparation was allowed 2 hours to reach a stable resting tension and during this time washed through with Krebs' solution in a continuous upward displacement.

For both the rabbit aorta and the guinea-pig trachea, two series of cummulative doses of the standard agonist, 11,9-epoxymethano PGH_2 were added with an intervening wash period of 60 minutes.

When studying the antagonistic action of thromboxane receptor blockers, the antagonist concerned was added directly to the bath and left in the bathing solution for 30-60 minutes (the flow of Krebs' solution was stopped during this period). The preparation was challenged with another series of cummulative doses (suitably increased) of 11,9-epoxymethano PGH_2. Only one antagonist was

studied in any one preparation.

At the end of each experiment, any changes in the sensitivity of the preparations were estimated by calculating the difference in responses of the control preparation at the beginning and end of the experiment. This difference was expressed as a fraction of the total initial response (correction factor). The agonist and antagonist potencies determined in other preparations were corrected for any changes in sensitivity by multiplying by this factor.

Ligand Binding Methodology

Both fresh human blood platelets and fresh platelet packs (obtained from the Blood Transfusion Unit, Royal Infirmary of Edinburgh) were used in the preliminary ligand binding studies carried out in this thesis. Platelet packs were less than 24 hours old.

A. Preparation of platelet membranes

Human blood (200ml) was centrifuged at 200 x g for 20 minutes. Prostacyclin (10 ng/ml final conc.) was added to the PRP which was spun for a further 20 minutes at 600 x g. The platelet pellet was resuspended in approximately 50ml of Tris buffer pH 7.4 (50 mM)-Proc. I

Fresh platelet packs (x 2) were spun down at 200 x g for 20 minutes to remove any residual red cells present. The platelet concentrate was diluted 1:1 with tris buffer pH 7.4 (50mM)-Proc. II. Both I and II were ultracentrifuged at 9000 x g for 15 minutes at $4^{o}C$ and

the pellet was resuspended in 5mM Tris Buffer. This suspension was homogenized with a Polytron homogenizer for 2-3 minutes, breaking up the cells. The lysed platelet suspension was centrifuged for a further hour on an ultracentrifuge at 120,000 x g at 4°C. The pellet was taken up in 16 mls of Tris Buffer (50 mM). If necessary, the suspension was homogenized for a few minutes to break up the pellet. The amount of protein present in the membrane suspension was determined by the method of Lowry (1951). The membrane suspension was adjusted with Tris Buffer (50mM) to give a final protein concentration of 4-6 mg ml^{-1}.

B. Binding Assay Protcol

Tubes were set up in triplicate, each assay tube containing the following:-

Tris Buffer pH 7.4 (50mM)	30µl
$Mg.SO_4$ in 50mM Tris buffer (100mM)	10µl
radioligand in 50mM Tris buffer (cont.5% etoH)	10µl
selected PG in 50mM Tris buffer (cont.5% etoH)	10µl
or buffer control (also cont. 5% etoH).	10µl
platelet membranes (protein content 0.16mg)	40µl
Final assay volume =	100µl

The tubes were set up as described above (omitting the membranes) and kept on ice throughout. At 40 second intervals 40µl of the membrane suspension was added to a tube which was whirlimixed, then incubated for 10 minutes at 37°C. The contents of the tubes

were filtered through Whatman GF/B glass filter discs, and washed a further two times with 3.5ml of ice cold Tris buffer (50mM). The filter papers were dried under a lamp for 1 hour and then placed in 10ml scintillant fluid I. The filters were counted for radioactivity on a Philips PW4540 liquid scintillation counter. (*See page 48).

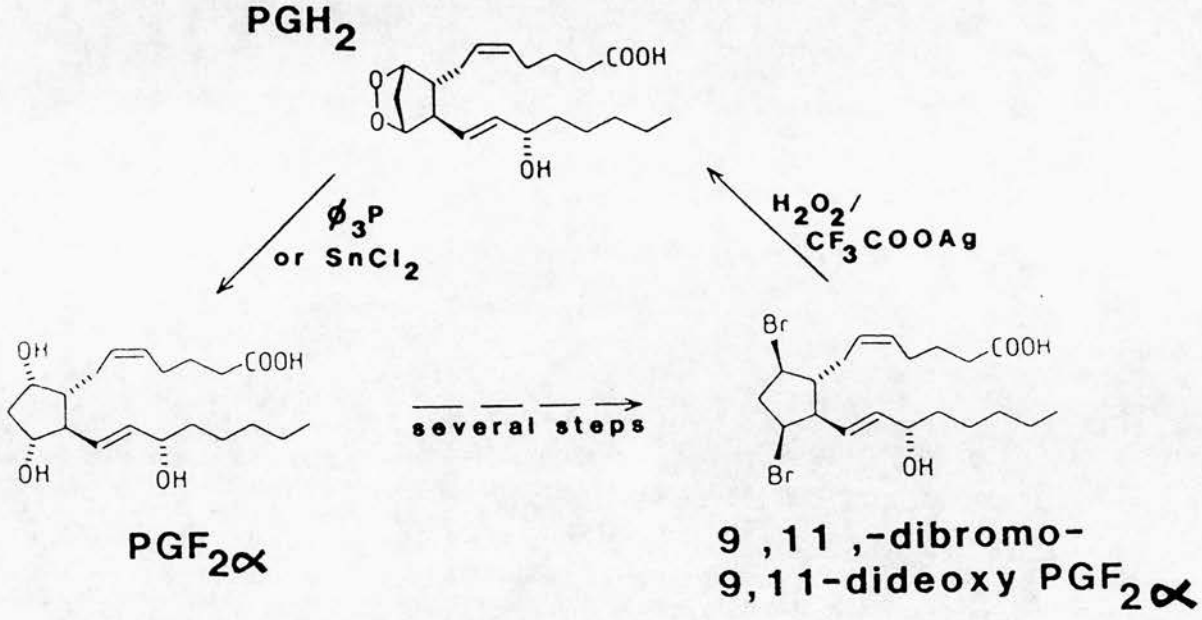
Preparation of Synthetic Prostaglandin H_2 (PGH_2)

$PGF_{2\alpha}$ was converted into 9β,11β-dibromo-9,11-dideoxy $PGF_{2\alpha}$ by the method of Porter et al (1979). Treatment of the dibromo derivative with 90% H_2O_2/Silver trifluoroacetate converted the dibromo derivative to PGH_2 which was purified by silicic acid chromatography (10g Unisil, 100 - 200 Mesh, Clarkson Chemical Co., U.S.A.; gradient elution, 20% ethylacetate in hexane to pure ethylacetate over 3 hr; - 20°C). Fig.2.6 illustrates this conversion of $PGF_{2\alpha}$ to PGH_2.

Biological Assay of PGH_2

PGH_2 activity was measured using a washed platelet system, where aggregation was the biological response.

A sample of the PGH_2 in ethylacetate (at -20°C) was dried down at 0°C on a rotary evaporatory and taken up in a volume of ice-cold Krebs' solution, and kept on ice throughout the experiment. An aliquot PGH_2 (in Krebs' solution) was added to a cuvette containing the washed platelet suspension, and incubated for 2 minutes at 37°C. The aggregation response observed gives a measure of the biological



$SnCl_2$	Stannous chloride
H_2O_2	Hydrogen peroxide
CF_3COOAg	Silver trifluoroacetate
$\emptyset_3P$	Triphenylphosphene

Figure 2.6 Synthesis of PGH_2 from dibromo - $PGF_{2\alpha}$ derivative and the conversion of PGH_2 to $PGF_{2\alpha}$ by stannous chloride.

activity of the PGH_2 and log concentration response curves were established to compare the activity of PGH_2 to other thromboxane mimetics.

A quantitative estimation of the mass of PGH_2 used in these experiments was made by reduction of approximately 1 µg of PGH_2 (50µl of PGH_2 in the Krebs' solution) to $PGF_{2\alpha}$ with fresh stannous chloride (0.5 mls of 10mg/ml solution) in aqueous ethanol. This mixture was left for 30 minutes at room temperature, before adding 5ml H_2O and then extracted with 2 x 5ml volumes of ethylacetate. The ethylacetate mixture was washed with 2ml H_2O. These samples (aqueous/ethylacetate) were frozen until ready for assay. Before assaying for $PGF_{2\alpha}$ the samples were thawed, the ethylacetate layer carefully removed and made up to 10 mg (11 mls) with ethylacetate. Aliquots of the $PGF_{2\alpha}$ samples in ethylacetate were prepared for radioimmunoassay.

The amounts of $PGF_{2\alpha}$ measured were corrected for decay of the PGH_2 during experimental procedures (30% loss per 2 hr).

* The separation of free and bound ligand by this filtration method took about 1 minute. The conditions were not optimalised and it is possible that the procedure could have been shortened if optimal conditions were found.

Solutions

Acid citrate dextrose (ACD) - 120ml

3g D-glucose	BDH Chemicals Ltd. U.K.
2g Disodium Hydrogen Citrate	"

Krebs' solution - 1 litre

2.1g Glucose	BDH Chemicals Ltd. U.K.
2.1g Sodium Dihydrogen Phosphate	"
6.9g Sodium Chloride	"
1.4ml Magnesium Sulphate (10%)	"
1.6 ml Potassium Dihydrogen orthophosphate (10%)	"
2.5 ml Calcium Chloride (M)	"
3.5 ml Potassium Chloride	"

E.D.T.A. Solution - ethylenediaminetetra-acetic acid BDH Chemicals Ltd. U.K.

0.4% weight/volume in 0.9% saline

Phosphate buffer pH 7.5 (.05M) - litre

6.9g diSodium hydrogen phosphate (anhydrous)	BDH Chemicals Ltd. U.K.
11.2ml Sodium hydrogen phosphate (: $2H_2O$) (1M)	"
0.1g sodium azide	Hopkin & Williams Ltd. U.K.
1.0g Gelatin	BDH Chemicals Ltd. U.K.

Tris Buffer pH 7.4 (500mM) - 1 litre

6.057g of Tris taken to pH 7.4 with concentrated HCl	May & Baker Ltd. U.K.

Magnesium Sulphate in tris buffer (100mM) - 1 litre

24.6g Magnesium Sulphate in tris	BDH Chemicals Ltd. U.K.
0.4g Bovine Serum Albumen	BDH Chemicals Ltd. U.K.

Cyclic AMP assay buffer - 1 litre

0.6g Tris buffer (50mM)

take to pH 7.5 with concentrated HCl.

1.48g E.D.T.A. (4mM)

Binding Protein

1.5ml Binding Protein	BDH Chemicals Ltd. U.K.
40ml cAMP assay buffer (above)	"

Scintillant Fluid I

10.5g 2,5-diphenyloxazole (PPO)	Fisons U.K.
1.5 litre Toluene	BDH Chemicals Ltd. U.K.
0.9 litre 2-ethoxyethanol	BDH Chemicals Ltd. U.K.

Scintillant Fluid II

P.C.S. Scintillant	(2 parts)	
Toluene	(1 part)	BDH Chemicals Ltd. U.K.

Chemicals and materials

1,2-Dichloroethane (HPLC grade)	Rathbone Chem. U.K.
Ethanol	J. Burroughs Ltd. U.K.
Ethylacetate (HPLC grade)	BDH Chemicals Ltd. U.K.
Glacial acetic acid (analar)	"
Hexane (analar)	"
Methanol (analar)	"
Petroleum spirit (b.p. 40-60°C Reagent grade)	"
Chloroform	Rathbone U.K.
Sodium Citrate	BDH Chemicals Ltd. U.K.
Mercuric Chloride	"
Brilliant Cresyl Blue	Pharmacia, Sweden
Urea	BDH Chemicals Ltd. U.K.
TXB_2 antibody	Institut Pasteur, France
DARS (1:10 dilution in diluent)	Scottish andibody Production unit, U.K.
NRS (1:40 dilution in diluent)	Prepared by Dr. N.L. Poyser by method of Dighe et al 1975.
^{3}H TXB_2 (sp.act. 6.66 TBq $mMol^{-1}$)	Amersham Int. U.K.
^{14}C-5HT (sp.act. 1.92 GBq $mMol^{-1}$)	"
^{3}H-Hexane (sp.act. 260 kBq)	"
^{3}H-cAMP (sp.act. 980.5 GBq $mMol^{-1}$)	"
Drugs used;	
Arachidonic Acid	Sigma Chem. Co. U.K.
Flurbiprofen	Boots Company U.K.

Iloprost	Schering AG
Prostacyclin	" "

Most authentic PGs, thromboxanes, their metabolites and stable analogues were gifts of Dr. J. Pike, Upjohn Co., Kalamazoo, U.S.A.

All EP compounds, 9,11 epoxymethano PGH_2 (labelled by Amersham U.K.) and PGH_2 were prepared by chemical synthesis in this department by Dr. N.H. Wilson.

CHAPTER 3

Arachidonic Acid Metabolism in Rat Platelets

INTRODUCTION

Variations in the anatomy and physiology of platelets from different species are well established (Dodds, 1978). Such differences may manifest themselves in the mechanisms underlying the arachidonic pathway since several studies have demonstrated species variations in reactivity of platelets to arachidonic acid. Chignard et al (1976) have shown that platelets from some dogs do not aggregate to arachidonic acid even though they form TXB_2. Meyers et al (1979) have demonstrated that equine platelets rarely exhibit biphasic aggregation responses and irreversible aggregation has not been observed with arachidonic acid. In addition Meyers (1980) has also shown lack of an aggregation response to arachidonic acid and thrombin in platelets from mink, pig and cow. Human platelets on the other hand have a well developed arachidonic acid pathway producing significant amounts of the arachidonate metabolites, malondialdehyde and thromboxane B_2 (Meyers et al, 1980). With the apparently significant role of arachidonic acid pathway in man and its possible involvement in platelet related disorders, thrombosis and thromboembolism, it is therefore important to have an appreciation of the role of this pathway in the activation of platelets from other species especially those used as animal models in the evaluation of potential antithrombotic drug prior to their implementation in man.

The arachidonic cascade comes into effect upon stimulation of cell receptors with the subsequent release of arachidonic acid from membrane phospholipids. The mobilization of arachidonic acid from

membrane phospholipids is the rate limiting step in the further metabolism of arachidonic acid to thromboxane and lipoxygenase products (Carey et al, 1985). The above sequence of events can be mediated in platelets by stimulation with certain substances; thrombin, collagen, ADP and adrenaline. In the case of thrombin, it has been established that AA is liberated from phosphatidylcholine in the platelet membrane and then converted to endoperoxides and thromboxanes (Bills et al, 1975). Collagen and ADP also induce prostaglandin formation during aggregation (Smith et al, 1972). The subsequent production of prostaglandin endoperoxides and thromboxane A_2 by these agents contributes to the second phase of aggregation and the release reaction, demonstrated by experiments using non-steroidal anti-inflammatory drugs (NSAID). O'Brien, (1968) using aspirin showed that the irreversible aggregation was inhibited when platelets were challenged with moderate amounts of thrombin and collagen, the primary wave was unaffected. ADP, although a weaker stimulant than thrombin or collagen also induced aggregation with the concomitant liberation of arachidonic acid.

The contributory role of arachidonic products in mediating the platelet response has been most thoroughly studied in the human model, but one should bear in mind that the precise role of the arachidonic cascade will undoubtedly vary from species to species. Controversy does exist as to the involvement of the arachidonic metabolites in mediating platelet aggregation in other species, (Dutihl et al, 1978; Bult and Bonta, 1976). Several groups have suggested the possible involvement of lipoxygenase products in the platelet response (Dutihl et al, 1978, 1980; Buchanan et al, 1986).

Overall, the arachidonic acid cascade, associated with platelet-aggregation and in particular prostanoid formation has been well documented (Samuelsson et al, 1978). The lipoxygenase pathway has received much less attention especially with respect to the biological effects of the lipoxygenase products.

The lipoxygenase and prostaglandin synthase complex represents the 2 major pathways for the utilization of arachidonic acid in blood platelets; oxygenation of arachidonic acid by the prostaglandin synthetase complex yields prostaglandins and related compounds whilst oxygenation of 12-lipoxygenase enzyme results in the production of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) which is subsequently reduced to the 12 hydroxy - derivative, 12-hydroxyeicosatetraenoic acid (12-HETE) by a glutathione dependent peroxidase (Siegel et al, 1979). 12-HPETE has been shown to be an inhibitor of human thromboxane synthetase enzyme (Hammarstrom et al, 1977) as well as inhibiting the liberation of arachidonic acid from phosphatidylinositol, the latter activity being shared by 15-HPETE (Rittenhouse - Simmons 1980; Lagarde et al, 1981B). More recently 12-HPETE has also been reported to counteract the aggregatory activity of PG endoperoxides/thromboxane A_2 (PGH_2/TXA_2) and a although significantly less potent, the reduced derivatives 12-HETE and 15-HETE exhibit similar inhibitory effects (Aharony et al, 1982).

Although the precise nature of the biological effects of the lipoxygenase products has yet to be clearly defined, one group have suggested a role for lipoxygenase products in platelet aggregation (Dutihl et al, 1980). Using rat blood, Dutihl and co-workers have

suggested that the products of the lipoxygenase pathway are essential for irreversible blood platelet aggregation during collagen induced aggregation. Using the dual cyclo-oxygenase/lipoxygenase inhibitor, eicosatriynoic acid (E.T.Y.A.), they found that there was a complete inhibition of collagen-induced aggregation. They also looked at concomitant product formation in the presence of E.T.Y.A. and demonstrated a decreased lipoxygenase activity, accompanied by an apparent increase in cyclo-oxygenase activity. Based on these results Dutihl suggested that for irreversible blood platelet aggregation, the activity of the lipoxygenase enzyme is possibly as essential as that of the cyclo-oxygenase. This suggestion was further supported by the observation that if one of these enzyme pathways is blocked, aggregation of rat blood platelets is always inhibited.

Furthermore, studies have also shown species differences in the contribution of arachidonic acid cascade in collagen-induced aggregation of platelets 'in vivo' (Mallarkey and Smith, 1985). Using the guinea-pig and rat model, Mallarkey and Smith demonstrated that arachidonic acid metabolites (prostaglandin endoperoxides and TXA_2) play only a minor role in collagen induced intravascular platelet aggregation in the rat (70% of the aggregation was independent of activation of the arachidonic acid cascade). On the other hand, conversion of arachidonic acid to PGH_2 and TXA_2 is of importance in the guinea-pig with 40% of the aggregation being independent of arachidonate conversion. These results demonstrate that species differences are observed 'in vivo' as well as 'in vitro' studies.

These differences highlight the need for elucidating the precise mechanisms involved in platelet aggregation in different species, since animal models are used in preclinical 'in vivo' and 'in vitro' studies to investigate drugs for potential anti-thrombotic therapy. Thus it is of prime importance to find such a model which bears a close resemblance to the human model with respect to it platelet mechanisms.

Since rats are frequently used in many platelet studies, this chapter deals with the elucidation of the precise nature of the arachidonic acid pathway and the role of its metabolites in platelet aggregation in rat blood. Much conflicting data exists concerning the nature of arachidonic acid induced aggregation in rat blood. Nishizawa et al (1983) has suggested that irreversible aggregation induced by arachidonic acid is not entirely dependent upon the formation of prostaglandin endoperoxides and thromboxanes A_2. Their studies demonstrated that both PGH_2 and the stable thromboxane mimetic, 11,9-epoxymethano PGH_2 failed to induce aggregation in citrated or heparinized platelet-rich plasma despite the formation of thromboxane B_2. However, full scale irreversible aggregation was demonstrated with arachidonic acid itself.

Work in our laboratory has also demonstrated a lack of irreversible aggregation using 11,9,epoxymethano PGH_2 and arachidonic acid in rat citrated platelet rich plasma. With both agents shape change did occur.

This study was aimed at clarifying the discrepancies observed in

other studies concerning the role of the arachidonic acid cascade, the contribution of prostaglandins and thromboxanes and the possible involvement of lipoxygenase products in inducing platelet aggregation.

The main aims of this chapter were as follows:-

i. to obtain a kinetic description of arachidonic acid activity in platelet rich plasma and washed platelet suspensions.

ii. to establish whether arachidonic acid itself or its metabolites induce shape change and the aggregatory response in platelets.

iii. and in light of the findings of (ii) to establish the relative importance of the prostaglandin endoperoxide, thromboxanes and the lipoxygenase pathway products in mediating the arachidonic acid responses.

Many studies have shown that variations in dietary fatty acids may alter the ratios of the fatty acid precursors incorporated into platelet membranes whereby the amount of arachidonic acid available is decreased. (Renaud et al, 1970). As a result the liberated unesterified fatty acids may be substrate for the lipopxygenase and/or the cycloxygenase routes. Consequently, the relative amounts of the fatty acids 'in vivo' may play a role in the regulation of arachidonic activity in platelets.

The final aim of this chapter was thus concerned with the effects of various fatty acids on arachidonic acid induced responses.

RESULTS

Citrated Rat Platelet Rich Plasma

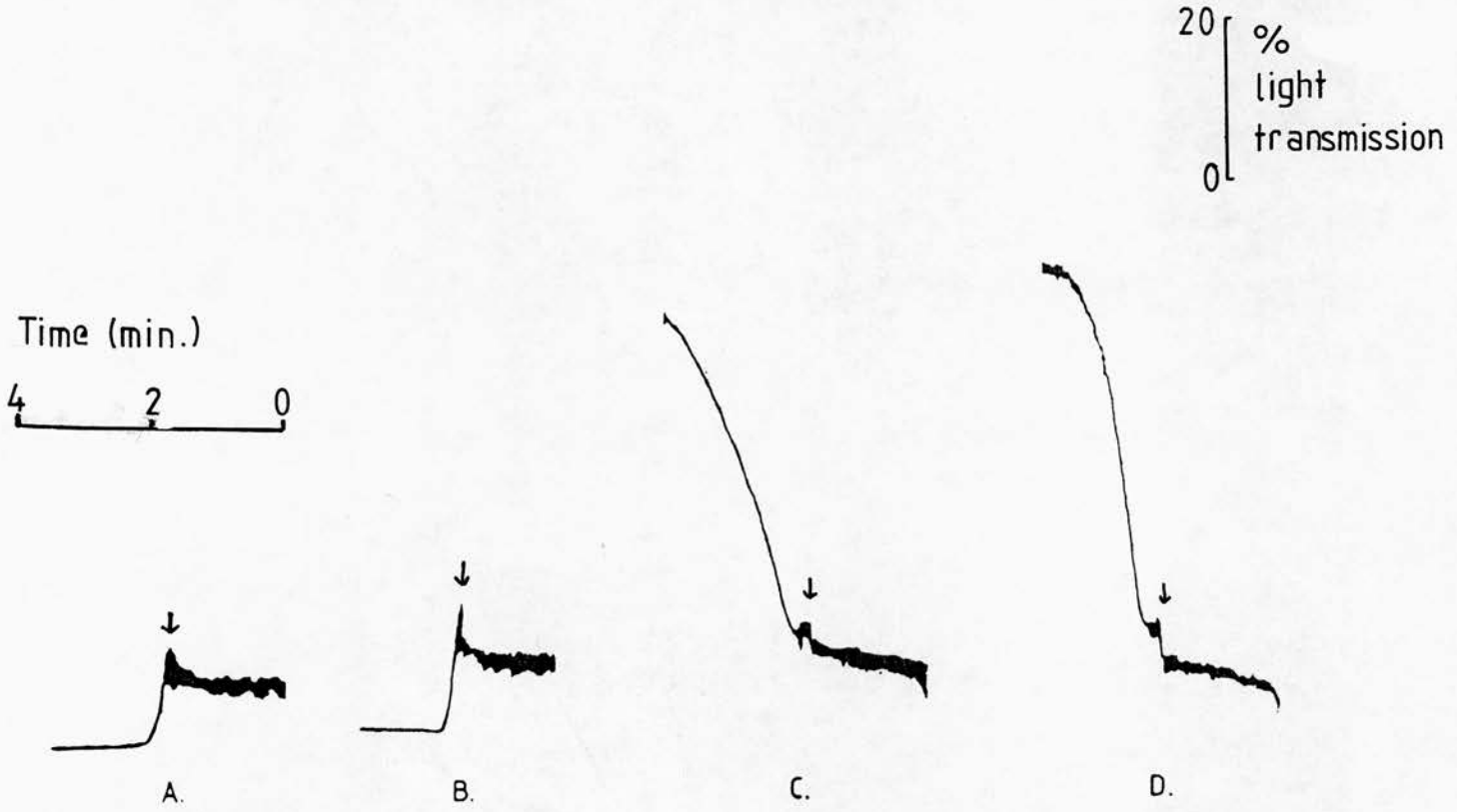
The responses of rat platelets in citrated plasma to stimulation with sodium arachidonate and two stable thromboxane mimetics, 11,9,epoxymethano prostaglandin H_2 (11,9-emPGH_2) and EP171 were studied.

The response of the platelets to stimulation with sodium arachidonate is shown in Fig.3.1. Shape change responses were observed with low arachidonate concentrations (10-250μM)and only when the arachidonate concentration was increased above 500μM was a maximal aggregation response observed. The effects of several blockers on these responses were investigated; Fig.3.2 illustrates the effect of the thromboxane receptor antagonist, EP092 (3μM), the cyclo-oxygenase inhibitor, Froben (10μM) and the thromboxane synthetase inhibitor, Dazoxiben (312μM) on the shape change response induced by AA (250μM). Both EP092 and Froben completely abolished the arachidonate induced shape change. The thromboxane synthetase inhibitor Dazoxiben on the other hand was found to only partially inhibit the response.

Abolition of the platelet response can also brought about by elevations in cyclic AMP levels through activation of platelet prostacyclin (PGI_2) receptors. The effects of both Iloprost, a stable prostacyclin mimetic (0.06μM) and PGE_1 (1.5μM) on arachidonate induced shape change were studied. Fig.3.2 illustrates the

Figure 3.1 Arachidonic acid induced responses in citrated rat PRP; (A) 10μM, (B) 250μM, (C) 500μM and (D) 1000μM arachidonic acid.

↓ drug addition


20
%
light
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Time (min.)
4
2
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A.
B.
C.
D.

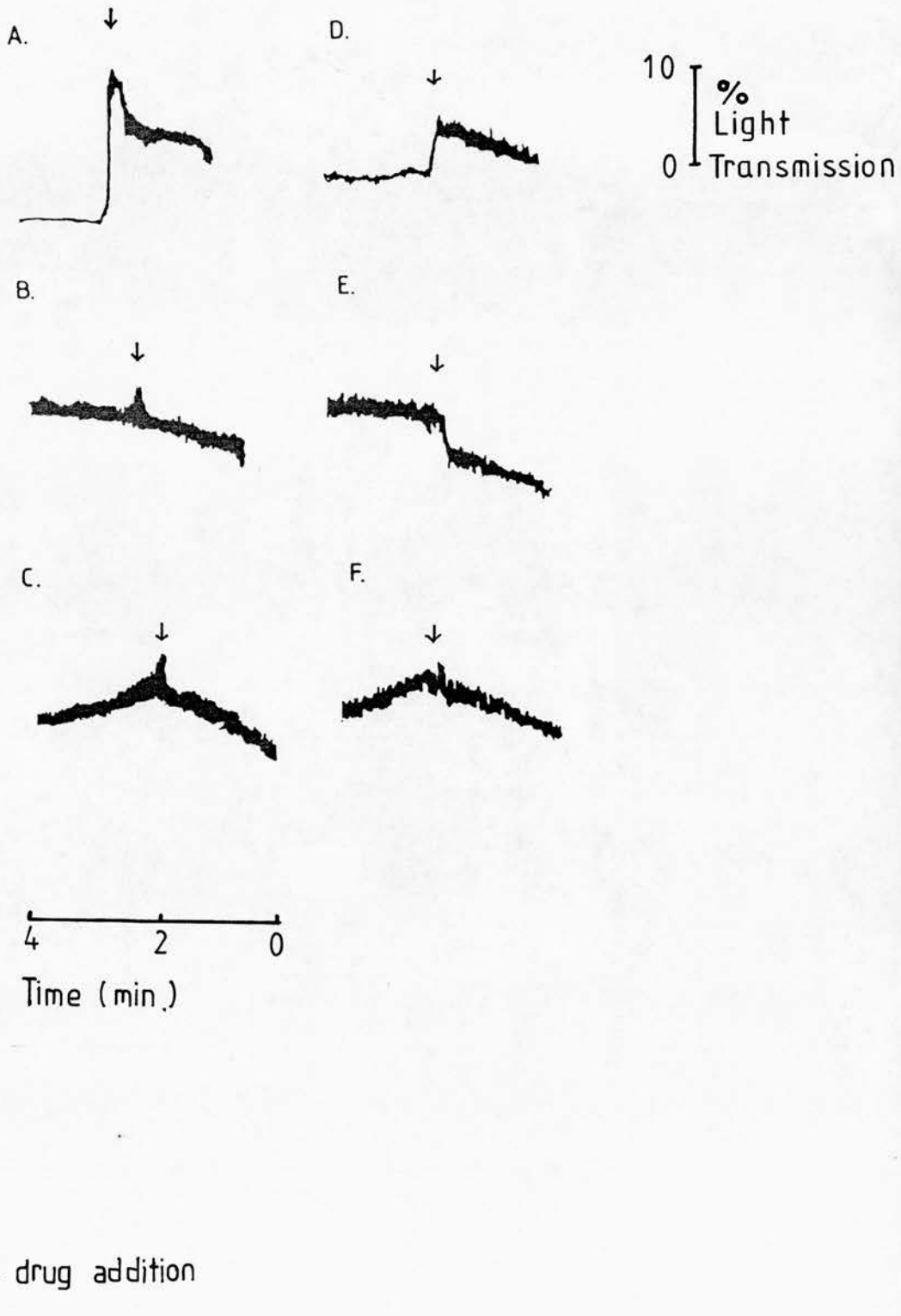


Figure 3.2 Effects of various blockers on arachidonic acid induced shape change in citrated rat PRP; (A) AA control (250μM) and in the presence of (B) EP092 (3μM), (C) Froben (10μM), (D) Dazoxiben (312μM), (E) PGE_1 (1.5μM) and (F) Iloprost (0.06μM).

inhibition observed with these prostaglandins.

The effects of two stable TX-mimetics 11,9emPGH$_2$ and EP171 were compared to those induced by arachidonate in rat platelet rich plasma. 11,9-emPGH$_2$ at concentrations up to 3μM produced only a shape change and EP171, although somewhat more potent than 11,9-emPGH$_2$ demonstrated a similar response at concentrations of 2.5-50nM. Increasing the concentrations of 11,9-emPGH$_2$ or EP171 over 3μM and 50nM respectively did not produce an irreversible aggregatory response (Fig.3.3A & D).

The effects of EP092 (6μM) Froben (10μM) and Dazoxiben (312μM) on 11,9-emPGH$_2$ and EP171 induced shape change were investigated. EP092 completely abolished the shape change response, whilst Flurbiprofen and Dazoxiben had little effect (Fig.3.3).

EP092 produced a parallel shift to the right of the shape change log dose response curve obtained with 11,9-emPGH$_2$ and EP171 (Fig.3.4). The apparent affinity constants for EP092 against EP171 and 11,9-emPGH$_2$ were determined from these curves and are shown in Table 3.1.

A comparison of the EP092 affinity constant for shape change in human platelet rich plasma using 11,9-emPGH$_2$ was made and is also shown in Table 3.1.

The nature of the irreversible aggregation induced by high arachidonate concentrations (>500μM) was investigated. Pre-

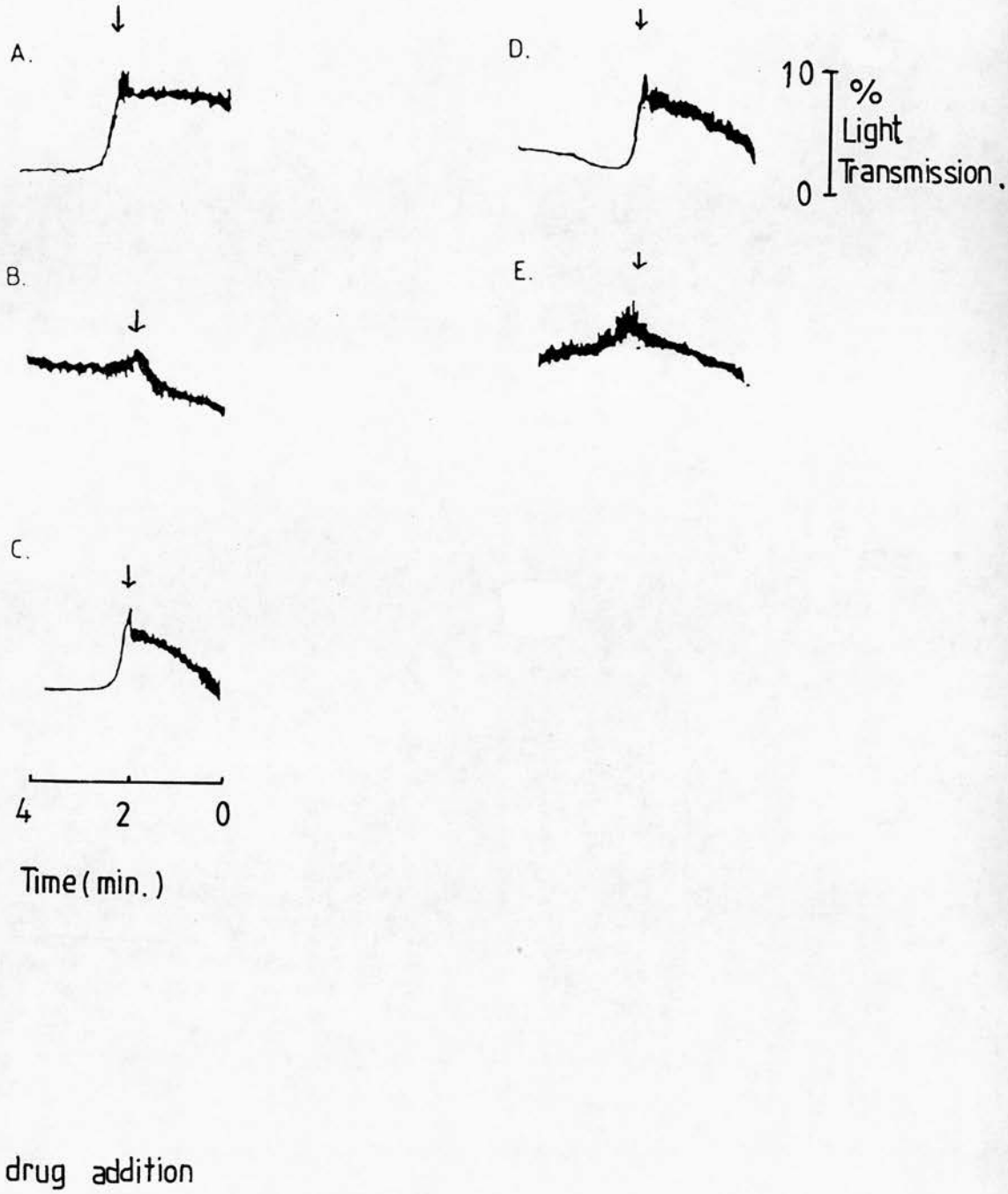


Figure 3.3 Shape change responses induced by 11,9-emPGH$_2$ and EP171 in citrated rat PRP; (A) 11,9-emPGH$_2$ control (300μM) and in the presence of (B) EP092 (6μM) and (C) Dazoxiben (312μM), (D) EP171 control (10nM) in the presence of (E) EP092 (6μM).

Figure 3.4 Log dose response curves for shape change induced by EP171 and 11,9-emPGH_2 alone and in the presence of EP092 (6μM) in citrated rat PRP.

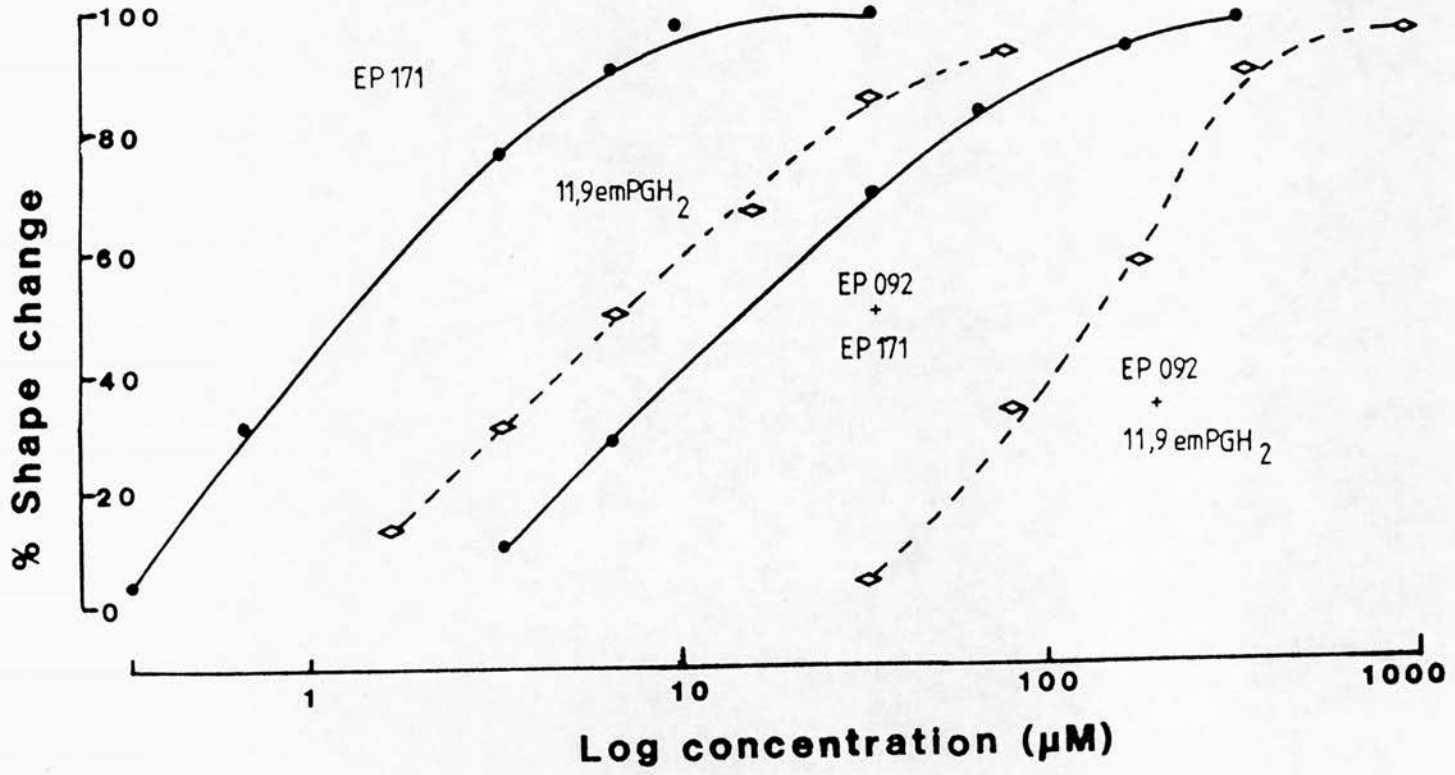

% Shape change
100
80
60
40
20
0
EP 171
11,9emPGH2
EP 092
+
EP 171
EP 092
+
11,9 emPGH2
1
10
100
1000
Log concentration (µM)

Table 3.1 The Affinity Constants for EP092 determined from 11,9emPGH$_2$ and EP171 induced shape change responses in rat citrated platelet rich plasma

	K_B value
11,9emPGH$_2$	3.04×10^6 (9.9×10^6)
EP171	6.48×10^6

Value in parenthesis obtained for EP092 against 11,9emPGH$_2$ in human PRP (Jones et al, 1985).

incubation with EP092 (15μM), Froben (10μM) and Iloprost (0.06μM) did not exert any significant inhibitory effect on this apparent irreversible aggregation (not shown).

Washed Rat Platelet Suspensions

Rat platelets suspended in plasma-free buffer aggregate irreversibly in response to micromolar concentrations of sodium arachidonate. The optimal concentration for this irreversible response being 10μM. On increasing the concentration of arachidonate to 100μM, the aggregatory response was supressed. However, at concentration of arachidonate over 30μM, what appeared to be an irreversible response was evident. Thus over a range of concentrations a bell-shaped response curve was produced with arachidonate (Fig.3.5). The mechanisms underlying each response were investigated and the results are reported below.

Irreversible Aggregation obtained with low AA concentrations

Arachidonate over a range of concentrations (1-30 μM) produces aggregation waves, a maximal response observed at 10μM (Fig.3.5A). The release of labelled 5HT (^{14}C-5HT) was associated with these aggregatory waves. EP092 (3μM), Froben (10μM) and Iloprost (0.06μM) all inhibited the aggregation induced by low micromolar concentrations of arachidonate.

Figure 3.5 Aggregation (continuous line) and [^{14}C]-5HT release (broken line) response of washed rat platelets to arachidonic acid.
Values displayed are means ± s.e.m. of 4 experiments.

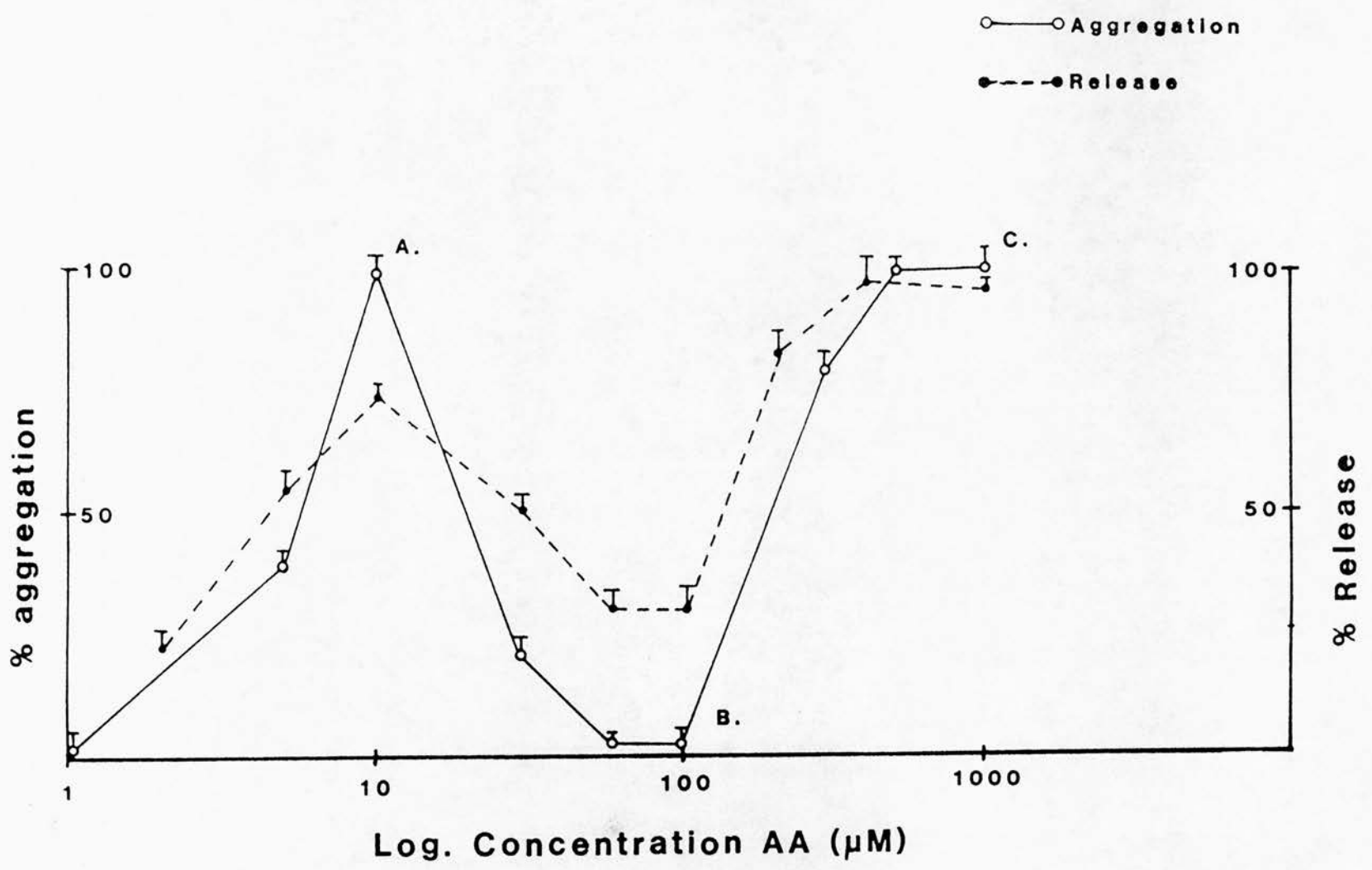

Aggregation
Release
A.
B.
C.
100
50
% aggregation
100
50
% Release
1
10
100
1000
Log. Concentration AA (μM)

Figure 3.6 Thromboxane B_2 production in response to increasing concentrations of arachidonic acid in washed rat platelets.
Values displayed are the mean ± s.e.m. of 4 experiments.

TXB2 (pg. per ml platelets)
1000
600
200
10
100
1000
Log. conc. AA (μM)

Suppression of aggregation intermediate arachidonate concentrations (100μM)

Over a very narrow range of AA concentrations (75-100μM) no aggregation response was observed (Fig.3.5B). It was noted that the responses of other aggregating agents in the presence of 100μM AA were also inhibited; ADP , 11,9-epoxymethano-PGH_2, Thrombin, collagen and low concentration of AA (Table 3.2). Thrombin at high concentration (1 unit ml^{-1}) could overcome this block.

The precise nature of this effect was investigated to see whether metabolites of either the cyclo-oxygenase or the lipoxygenase pathways or even arachidonate itself were mediating this inhibitory response.

Fig.3.7 illustrates the inhibitory effect of 100μM arachidonate on ADP induced aggregation. In the presence of froben (10μM) the inhibition of ADP (10^{-5}M) induced aggregation was not overcome (Fig.3.7C). Froben alone did not have any effect on the ADP aggregating wave (Fig.3.7D). A similar inhibition by AA (100μM) was observed when 11,9-epoxymethanoPGH_2 (700nM) was used as the aggregatory agent.

In order to investigate the possible role of lipoxygenase products as mediators of this inhibitory effect, the effects of several lipoxygenase inhibitors were studied.

No specific inhibitor for this enzyme is known as yet, and those

Table 3.2 Effect of 100μM AA on the action of other aggregating agents

AA (100μM) was incubated for 2 min before the addition of the following agents:-

AGENT	CONCENTRATION	% INHIBITION
ADP	1 μM	98%
	10 μM	78%
Collagen	2 μg/ml	100%
	4 μg/ml	96%
Thrombin	0.1 U/ml	100%
	1 U/ml	24%
11,9-epoxymethano PGH_2	1.5 μM	100%
	3 μM	100%
AA	10 μM	100%

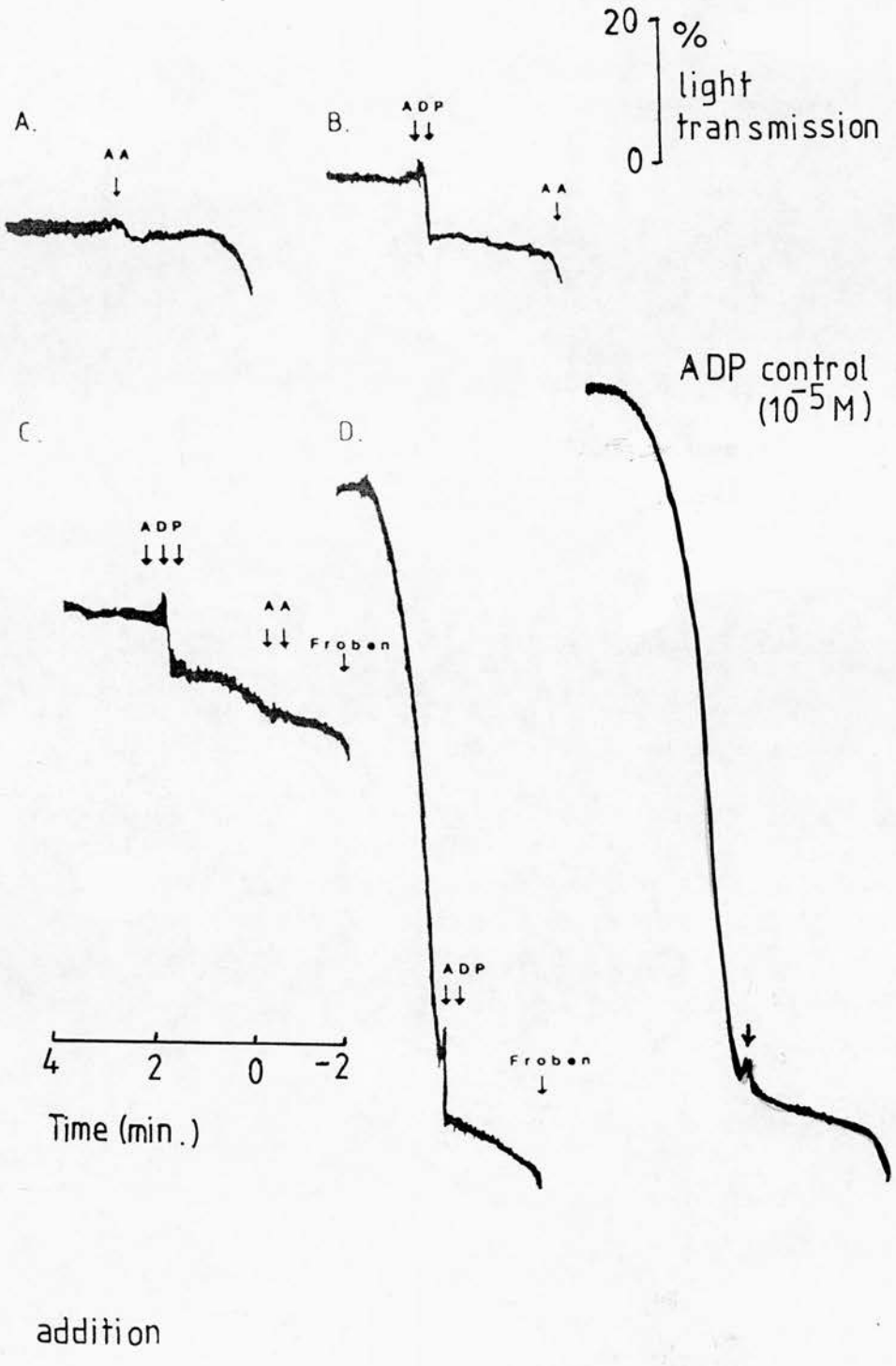


Figure 3.7 The inhibitory effect of pre-incubation with arachidonic acid (100μM) on ADP (10^{-5}M) induced aggregation in washed rat platelets and the effect of Froben (10μM) on this inhibition; (A) AA control (100μM), (B) AA (100μM) and ADP (10^{-5}M), (C) Froben (10μM), AA (100μM) and ADP (10^{-5}M) and (D) Froben (10μM) and ADP (10^{-5}M). Both AA and Froben were pre-incubated for 2 minutes prior to the addition of ADP.

studied in this chapter, were also inhibitors of the cyclo-oxygenase enzyme. Since it would appear that products of the cyclo-xygenase pathway are not mediators of the inhibition, dual inhibition of the lipoxygenase and cyclo-oxygenase enzymes should still demonstrate whether lipoxygenase products are mediators.

Rat washed platelet suspensions were pre-incubated with the lipoxygenase enzyme inhibitors, and their effect on the AA (100μM) inhibition was studied;

Nordihydroguaiaretic Acid (NDGA)

NDGA (33μM) inhibited the thromboxane/endoperoxide dependent irreversible aggregation attained with low AA concentrations (10μM) (not shown). There did not appear to be any reversal of the inhibitory activity of AA (100μM) on ADP (10^{-5}M) induced aggregation (Fig.3.8A-D) when platelets were pre-incubated with NDGA (33-165μM). The irreversible aggregation observed with AA at concentrations over 300μM appeared to be slightly inhibited in the presence of high NDGA concentrations (165μM) (Fig.3.8E and F).

Concentrations of NDGA over 180μM tended to directly affect the platelets causing them to change shape and induce small reversible aggregation waves, thus limiting the working concentration range of NDGA (1-165μM).

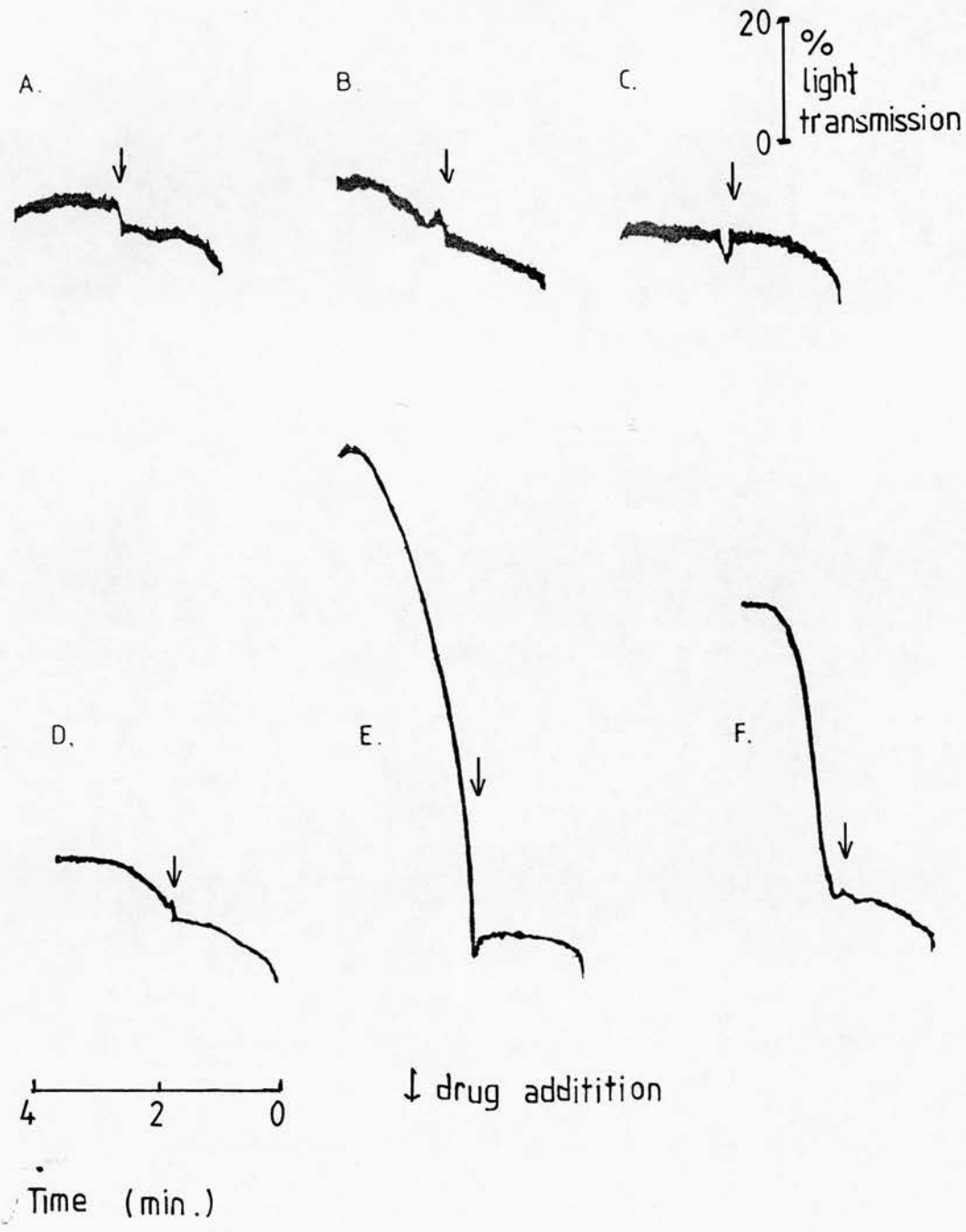


Figure 3.8 The effect of pre-incubation with NDGA on arachidonic acid induced responses in washed rat platelets; (A) NDGA (33μM) and AA (100μM), (B) AA (100μM) and ADP (10^{-5}M), (C) NDGA (33μM) and AA (100μM) and ADP (10^{-5}M), (D) NDGA (165μM), AA (10μM) and ADP (10^{-5}M), (E) AA (500μM) and (F) NDGA (165μM) and AA (500μM). NDGA and AA were pre-incubated simultaneously for 2 minutes prior to the addition of ADP (experimental traces run from right to left).

8,11,14-eicosatrienoic acid (E.T.A.)

E.T.A. (100μM) like NDGA inhibited the irreversible aggregation induced by low AA concentrations. This same concentration (100μM) did not have any effect on the inhibition of ADP (10^{-5}M) induced aggregation by AA (100μM). Furthermore, this concentration of E.T.A. did not have any effect on the irreversible wave attained with high arachidonate concentrations (300μM).

5,8,11,14 Eicosatetraynoic Acid (E.T.Y.A.)

E.T.Y.A. at concentrations between 3-5μM inhibited the irreversible aggregation induced by low AA concentrations. E.T.Y.A. (10μM) did not appear to reverse the inhibitory effect of 100μM AA on 11,9-emPGH$_2$ (750nM) and ADP (10^{-5}M) induced aggregations. However, pre-incubation with E.T.Y.A. alone (10μM) was found to inhibit both ADP and 11,9-emPGH$_2$ induced aggregation responses (Fig.3.9).

Higher E.T.Y.A. concentrations (15-30μM), were found to reverse the suppressed aggregation obtained with 100μM AA. However, pre-incubation with E.T.Y.A. in the presence of EP092 (3μM) and froben (10μM) did not appear to inhibit this irreversible wave (Fig.3.10).

BW755C

BW755C like all 3 other inhibitors, blocked irreversible aggregation induced by low arachidonate concentrations. Concentrations of BW755C (40-300μM) did not reverse the AA induced

Figure 3.9 The effect of pre-incubation with 5,8,11,14-ETYA on the inhibition by arachidonic acid of 11,9-emPGH$_2$ and ADP induced aggregation; (A) ETYA (10μM), AA (100μM) and 11,9-emPGH$_2$ (280μM), (B) ETYA (10μM) and 11,9-emPGH$_2$ (280μM), (C) ETYA (10μM), AA (100μM) and ADP (10^{-5}M) and (D) ETYA (10μM) and ADP (10^{-5}M).
Experimental traces run from right to left.

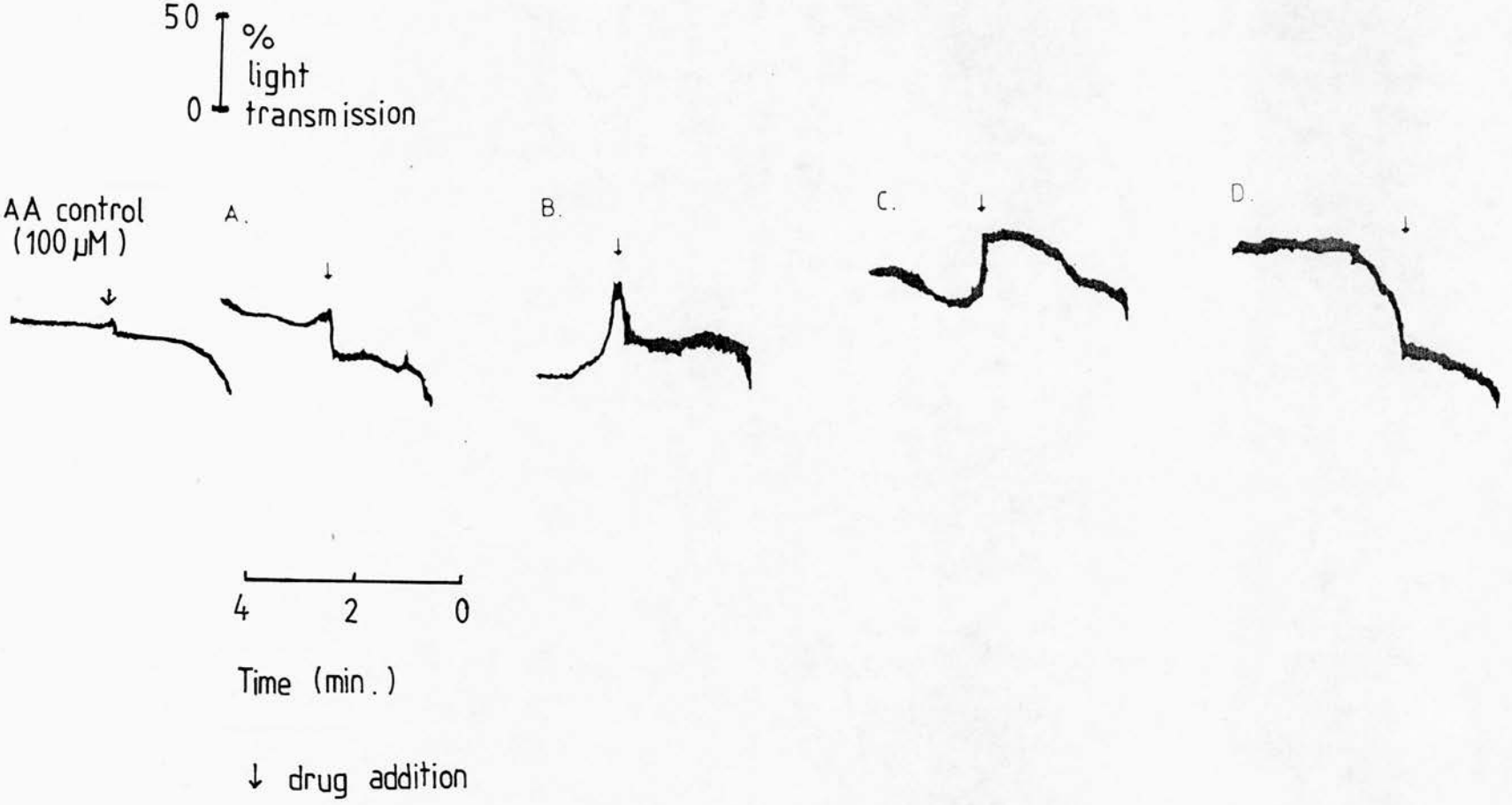
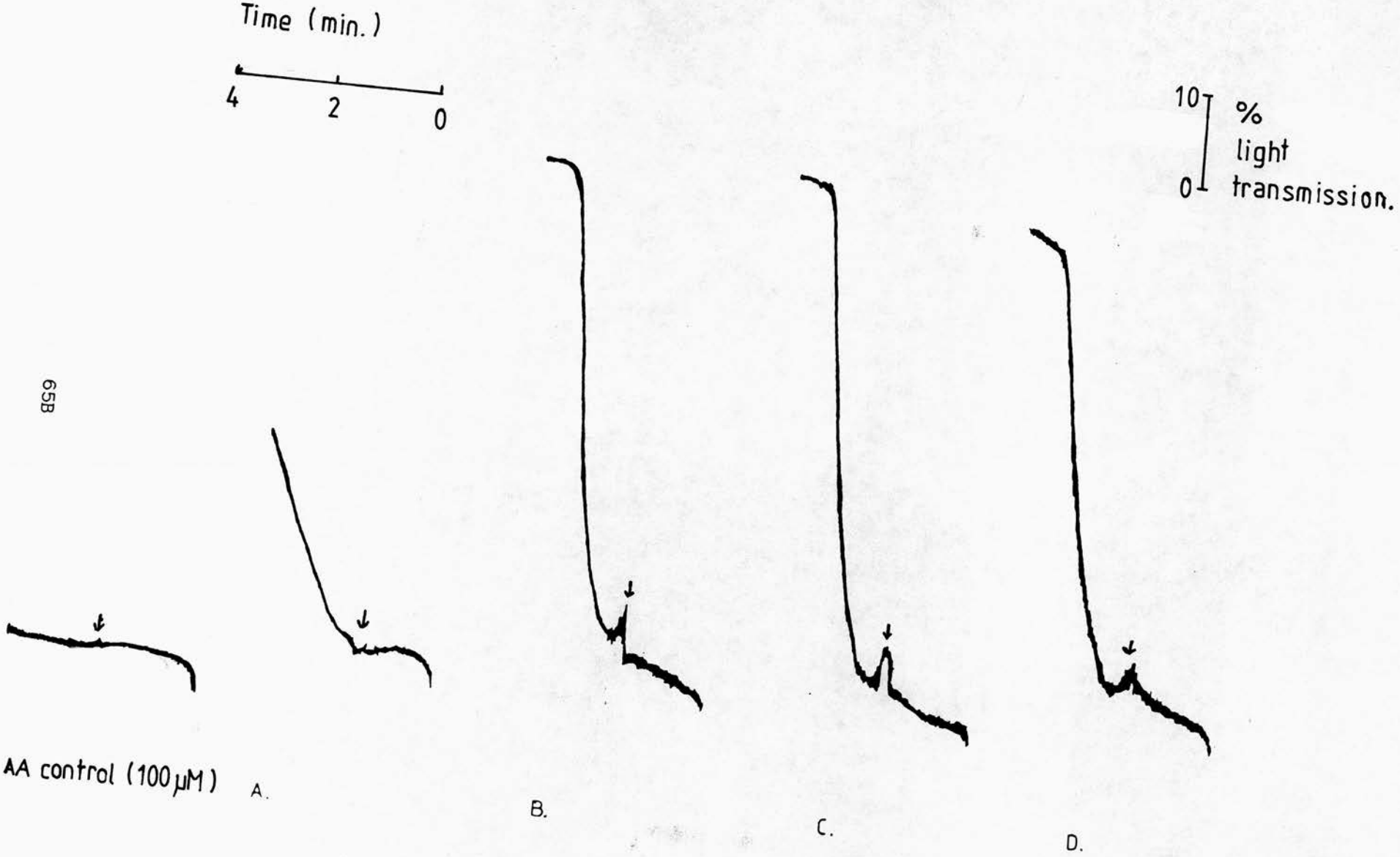

50
%
light
transmission
0
AA control
(100 µM)
A.
B.
C.
D.
4
2
0
Time (min.)
↓ drug addition

Figure 3.10 The effect of higher concentrations of 5,8,11,14-ETYA on the inhibition induced by arachidonic acid; (A) ETYA (15μM) and AA (100μM), (B) ETYA (20μM) and AA (100μM), (C) EP092 (3μM), ETYA (15μM) and AA (100μM) and (D) Froben (10μM), ETYA (15μM) and AA (100μM).
EP092 and Froben were added simultaneously with ETYA two minutes prior to the addition of arachidonic acid.

↓ drug addition


Time (min.)
4
2
0
10
%
light
0
transmission.
AA control (100 µM)
A.
B.
C.
D.

inhibition of ADP (10^{-5}) or 11,9-emPGH$_2$ (750nM). It was noted however that although aggregation induced by ADP (10^{-5}M) was unaffected by BW755C (100μM) the aggregation wave induced by 11,9emPGH$_2$ was completely abolished (Fig.3.11).

Table 3.3 summarizes the effects of the lipoxygenase inhibitors, reported above.

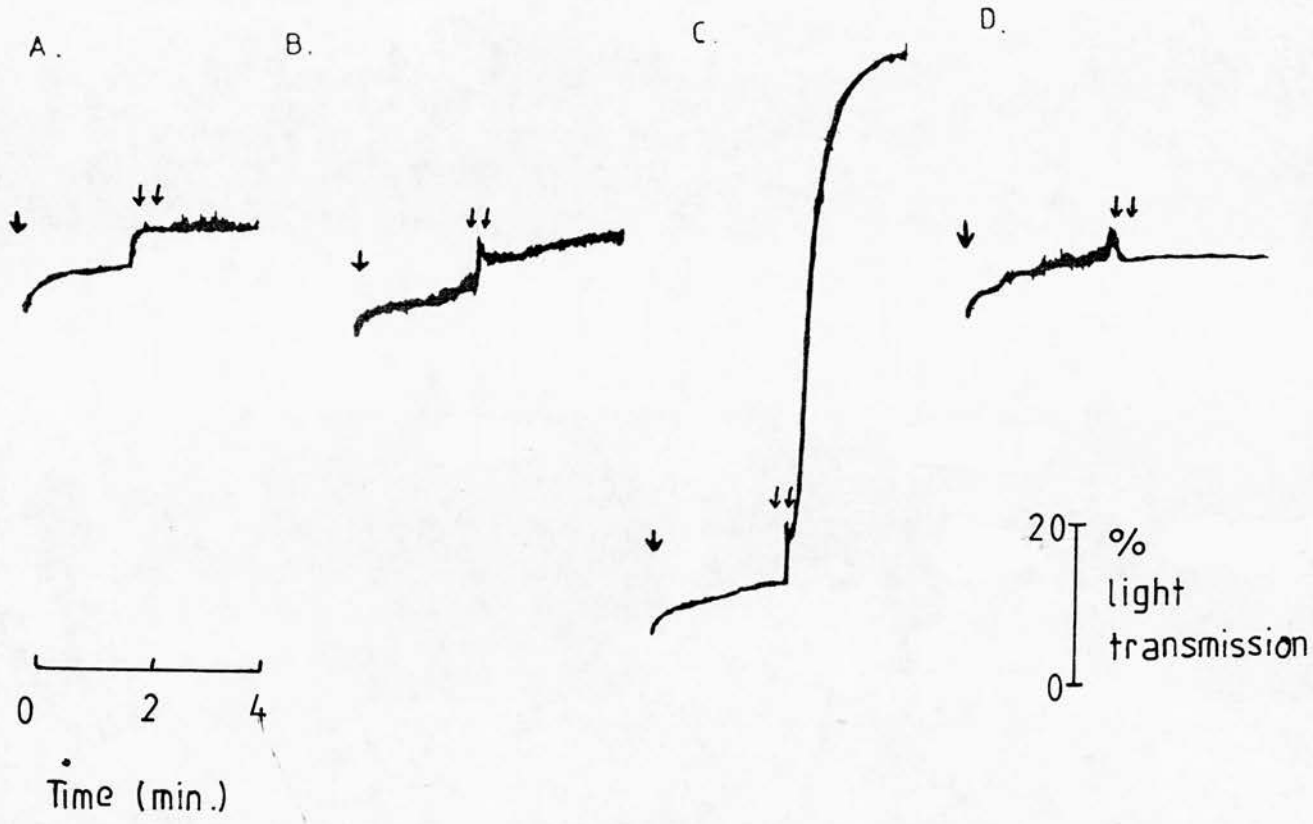
Time course of onset of inhibitory effect of AA (100μM)

A time course of the inhibitory effect of 100μM AA was studied to determine how soon following the addition of AA, the onset of the inhibition was apparent. Fig.3.12 illustrates this time course and within 10 seconds the inhibitory action has already come into effect, shown by the percentage inhibition of ADP (10^{-5}M) induced aggregation. Within 40 seconds as much as 80% of the ADP response is inhibited and complete inhibition is attained within 120 seconds.

The response of ADP (10^{-5}M) 15 minutes after the addition of 100μM AA was tested and the inhibitory effect was still fully evident after this time.

Maximal aggregation observed with high arachidonate concentrations (>300μM)

When arachidonate concentrations were increased over 300μM, a maximal aggregation response was observed, similar in appearance to an irreversible secondary wave aggregation response. The nature of



↓ drug addition

Figure 3.11 The effect of BW755C on the inhibitory effect of arachidonic acid; (A) BW755C (100μM), AA (100μM) and ADP (10^{-5}M), (B) BW755C (100μM), AA (100μM) and 11,9-emPG$_{H2}$ (750nM), (C) BW755C (10μM) and ADP (10^{-5}M) and (D) BW755C (10μM) and 11,9-emPGH$_2$ (750nM). BW755C and AA↓were added simultaneously 2 minutes prior to the addition of the aggregating agent↓↓. Experimental traces run from left to right.

Table 3.3 Effects of lipoxygenase/cyclo-oxygenase inhibitors on arachidonate induced responses in washed rat platelet suspensions

Inhibitor	Arachidonate concentration 10μM	100μM	0.5-1mM	Comments
N.D.G.A.				
(33μM)	full inhibition	no effect	no effect	
(165μM)	"	"	slight inhibition	produces shape change & small 1° wave aggregation at conc. over 165μM
8,11,14 E.T.A.				
(100μM)	full inhibition	no effect	no effect	produces small 1° wave aggregation at >100μM conc. of inhibitor.
5,8,11,14 E.T.Y.A.				
3μM	full inhibition	no effect	N.T.	ADP ($10\text{-}5_M$) and $11,9emPGH_2$ (750nM) aggregation inhibited at conc. > 10μM
15μM	"	reversal	N.T.	
30μM	"	reversal	N.T.	
BW755C				
40μM	full inhibition	no effect		$11,9emPGH_2$ (750nm) induced aggreg. inhibited
300μM	"	no effect		

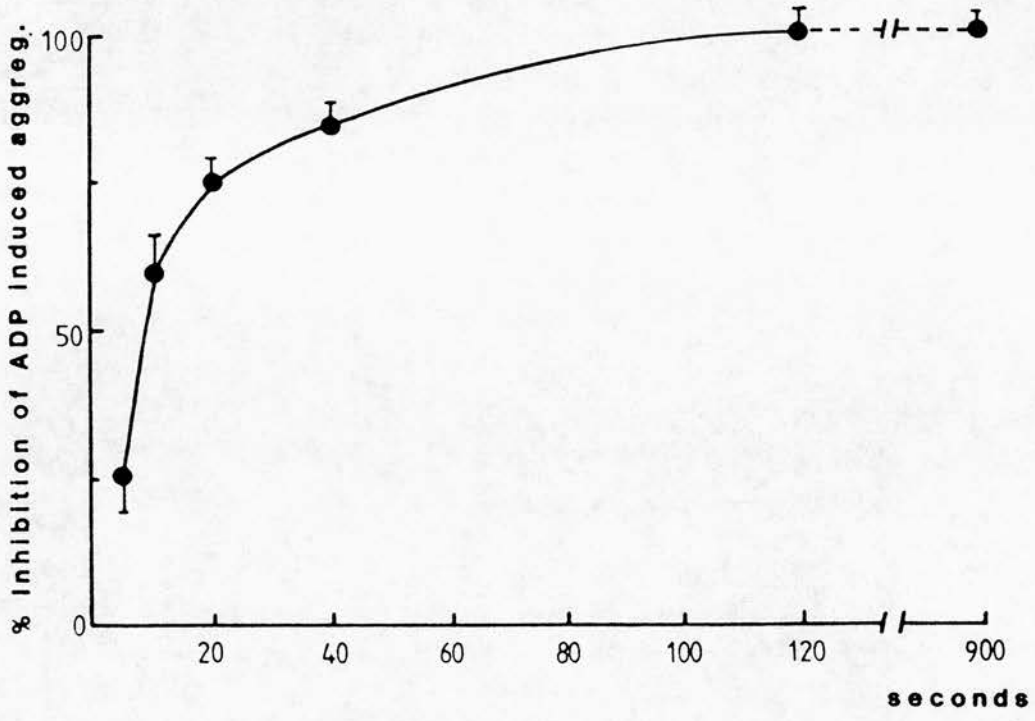


Figure 3.12 The time course of onset of arachidonic acid induced inhibition of ADP (10^{-5}M) aggregation. Arachidonic acid was added to the washed platelet suspension before the addition of ADP at the times indicated above.

this effect was investigated; Froben (10μM), EP092 (10μM) and Iloprost (0.06μM) had no inhibitory effect on this maximal response.

Thromboxane B_2 production, cyclic AMP production and 5HT-release in washed rat platelets measured over a range of arachidonic acid concentrations

TXB_2 production was measured in washed rat platelets suspensions over a range of arachidonate concentrations. Fig.3.6 illustrates a dose dependent increase in TXB_2 production, reaching a plateau at about 100μM arachidonate. On increasing concentrations of arachidonate over 100μM, TXB_2 production was maintained.

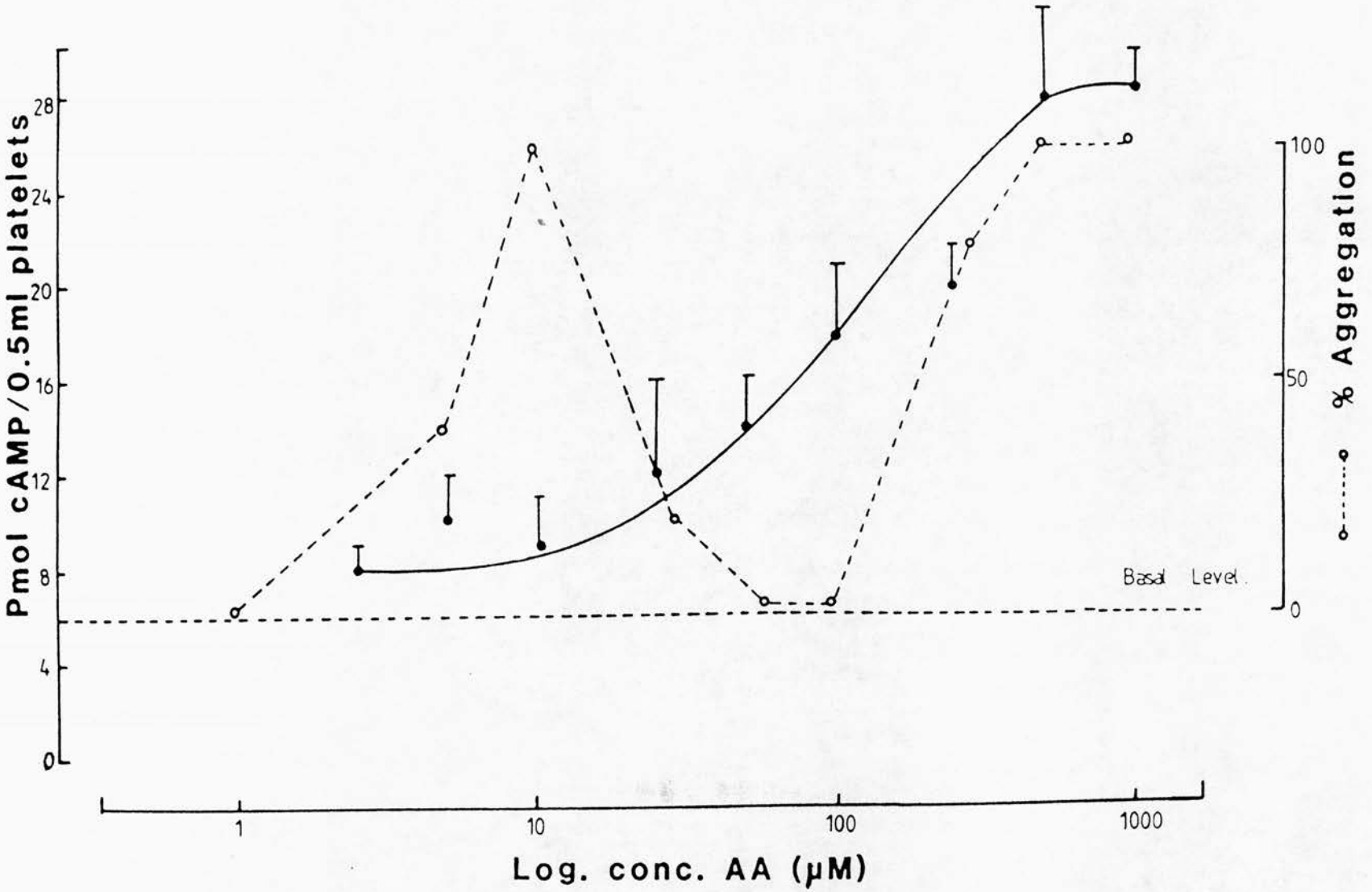
Cyclic AMP production was found to increase above basal levels from 50μM arachidonate concentrations upwards, and high levels were maintained over 300μM (Fig.3.13).

Simultaneous measurement of aggregation and [^{14}C]-5HT release was recorded in response to AA over a range of concentrations (1-1000μM). The concentration response curves for aggregation and release 5HT are shown in Fig.3.6. Maximal release is apparent at both peaks (10μM and 300μM) in the AA concentration-response curves.

The effect of linoleic acid and oleic acid effects on washed platelet suspensions

Linoleic acid at low concentrations (10μM) appeared to have no effect on washed platelet suspensions. However, in the presence of

Figure 3.13 The effects of arachidonic acid on platelet cyclic AMP levels. The data shows the concentration dependent effects of arachidonic acid on platelet aggregation and on cyclic AMP levels. (Cylic AMP levels were measured from samples following aggregation experiments). Values at each point correspond to the mean ± s.e.m. of 4 observations.


Pmol cAMP/0.5ml platelets
0
4
8
12
16
20
24
28
Basal Level
100
50
0
% Aggregation
1
10
100
1000
Log. conc. AA (μM)

higher linoleic acid concentrations (100μM) aggregation waves induced by ADP (10^{-5}M), 11,9-emPGH$_2$ (0.15-2.86μM) and Thrombin (0.1u/ml) were completely inhibited. Higher concentrations of Thrombin (1u/ml) could overcome this block. Increasing the concentration of linoleic acid over 500μM demonstrated that linoleic acid itself could induce irreversible waves (Fig.3.14) and these were associated with the release of ^{14}C-5HT.

Oleic acid over a range of concentrations (10-100μM) did not cause the platelets to aggregate but did inhibit the aggregation response to ADP (10^{-4}-10^{-6}M). The working concentration range of oleic acid was limited since it was difficult to get oleic acid into solution over 100μM.

These effects of both linoleic acid and oleic acid are summarised in Table 3.4.

Effect of fatty acid derivatives on AA induced responses in rat citrated PRP

Five ~~polyunsaturated~~ fatty acids were studied in citrated rat PRP, (structures are shown in Fig.3.15); 3,5,8,11,14 eicosapentanoic acid (20:5 W3); 5,8,11,14 eicosatetraynoic acid (20:4 YW6); 5,8,11-eicosatrienoic acid (20:3 W9); (docosatetraenoic acid C22:4 W6) and docosahexaenoic acid (C22:6).

The C22:6 (200-300μM) fatty acid caused the platelets to undergo shape change, and over 400μM small primary aggregation waves were

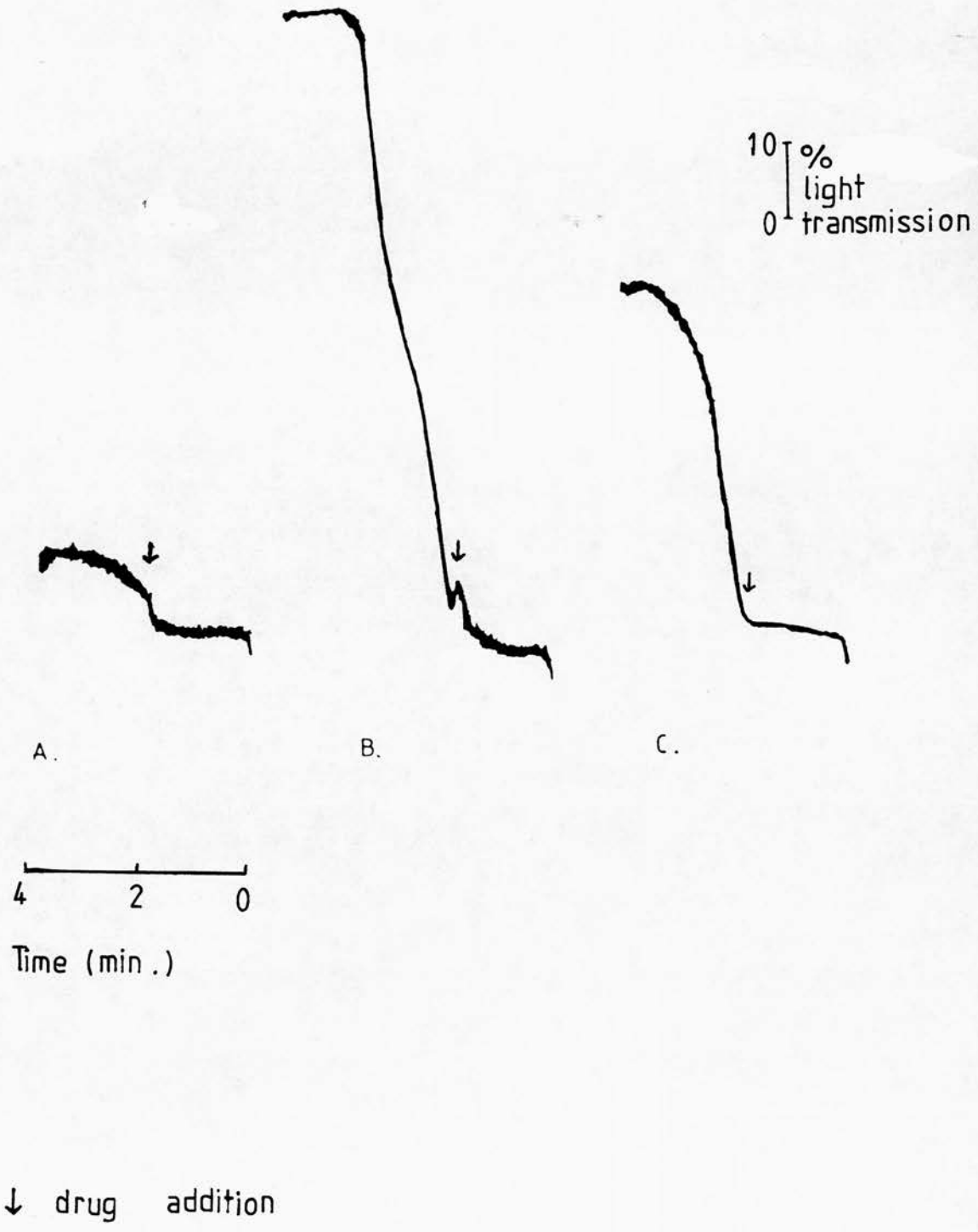


Figure 3.14 The effect of linoleic acid on thrombin induced aggregation; (A) linoleic acid (10μM) and thrombin (0.1U/ml), (B) linoleic acid (10μM) and thrombin (1U/ml) and (C) linoleic acid (500μM).

Table 3.4 Effects observed with linoleic and oleic acid in washed rat platelet suspensions

Fatty Acid	Concentration 10μM	100μM	0.5-1mM
Linoleic Acid	no effect on platelets	inhibited aggreg. induced by other agents; ADP (10^{-5}M) 100% inhibition Throm.(0.1U/ml) 100% inhibition 11,9 emPGH$_2$ (2.8μM) 100% inhibition	irreversible aggregation associated with the release reaction
	10μM	50μM	100μM
Oleic Acid	no effect on platelets	no aggregation	no aggregation
Inhibition of ADP-induced aggregation;			
ADP (10^{-6}M)	91% inhibition	100% inhibition	100% inhibition
(10^{-5}M)	51% "	100% "	50% "
(10^{-4}M)	51% "	53% "	10% "

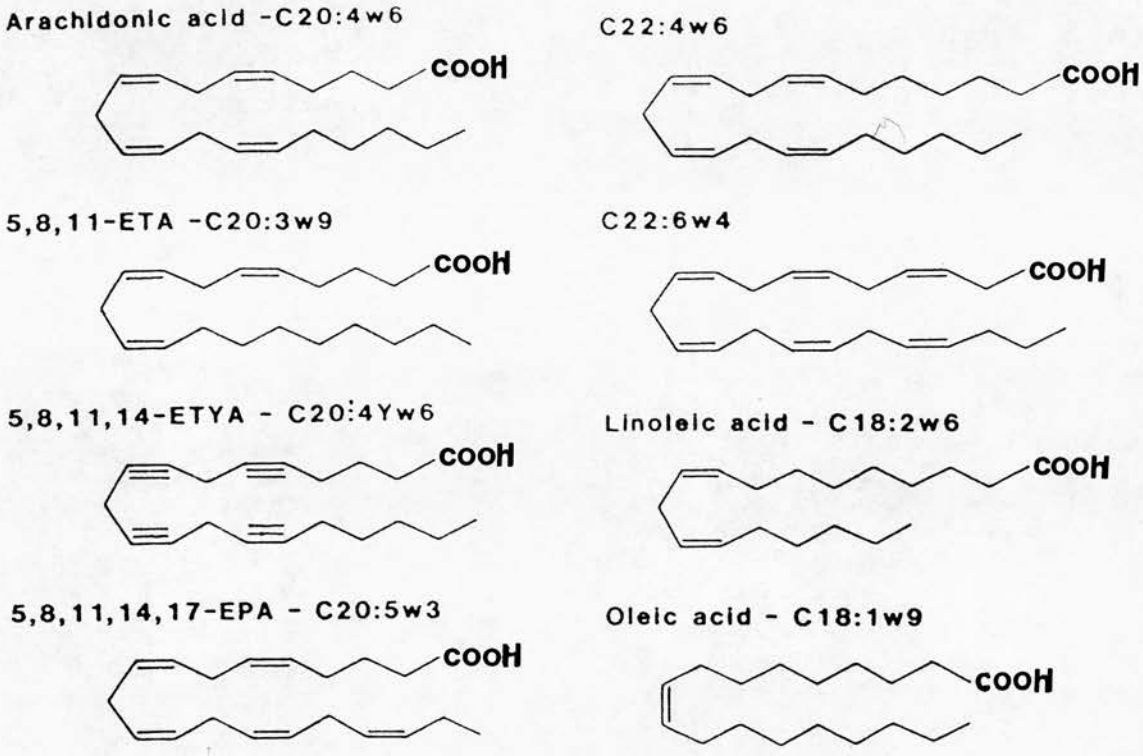


Figure 3.15 Structures of fatty acid derivatives used in this study.

apparent (Fig.3.16A). C22:4 W6, (400μM) did not have any apparent effect on the platelets themselves, but partially inhibited the shape change response induced by AA over 5-50μM concentration range. In the presence of C22:4W6 at concentrations of AA over 50μM, the concentration response curve was shifted to the left (Fig.3.16B). C22:5W6, (500μM) did not have any direct effect on the platelets themselves, but completely inhibited the shape change response induced by AA (5-100μM) and shifted the irreversible wave induced by AA slightly to the left (Fig.3.16C). C20:4YW6 (500μM) also completely abolished the shape change response induced by AA (5-100μM) and shifted the AA induced irreversible wave further to the left (Fig.3.16D). 20:3W6 was found to exert mixed effects on the AA induced responses. Low concentrations of 20:3W6 (100μM) had very little effect on the platelet shape change response, although a significant inhibition of the irreversible was apparent (Fig.3.17A). Higher 20:3W6 concentrations (500μM) completely inhibited the AA-induced shape change response, and significantly inhibited the irreversible wave (Fig.3.17B). 20:3W6 (1mM) also completely abolished the shape change response but only a small inhibition of the irreversible wave was apparent (Fig.3.17C).

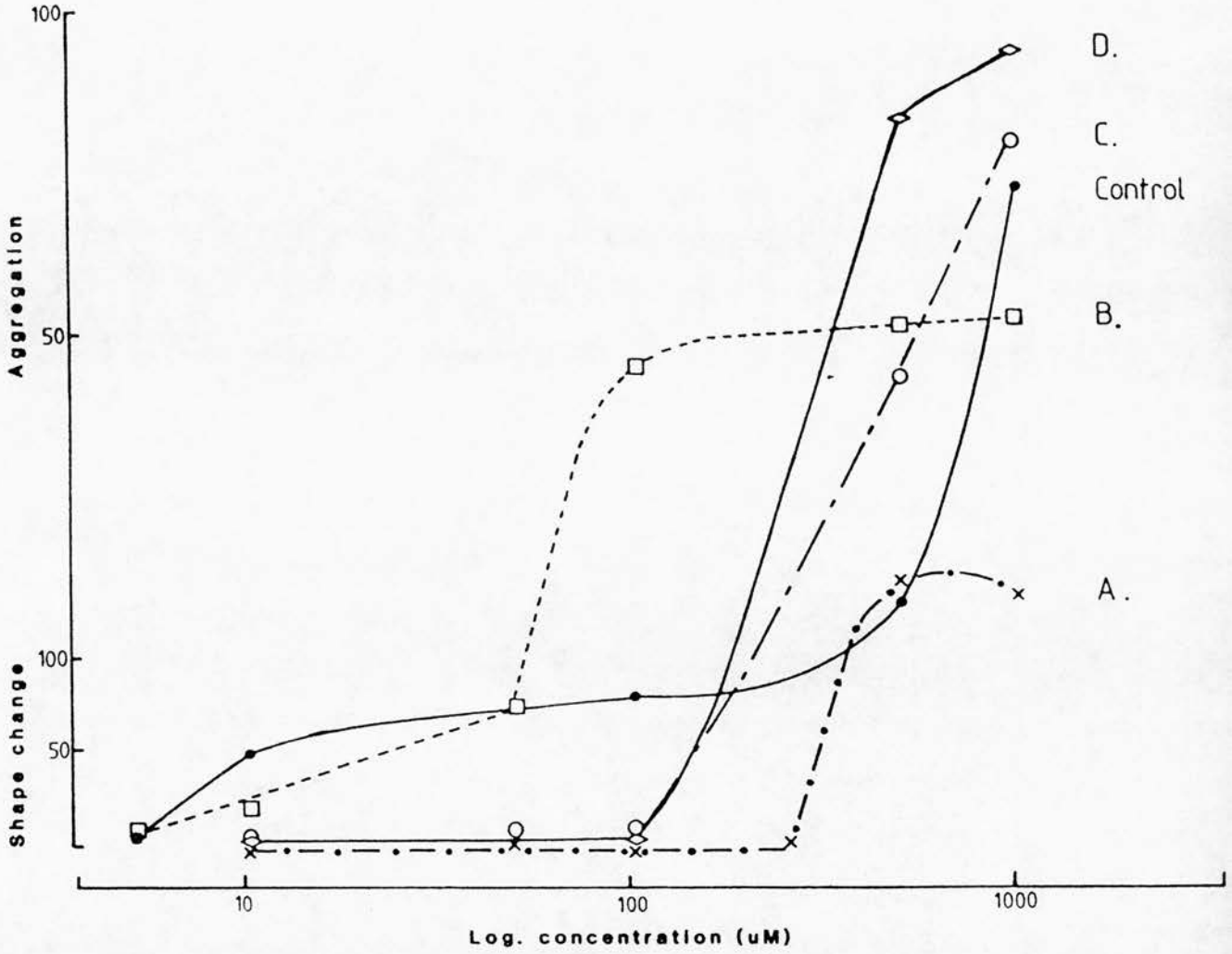
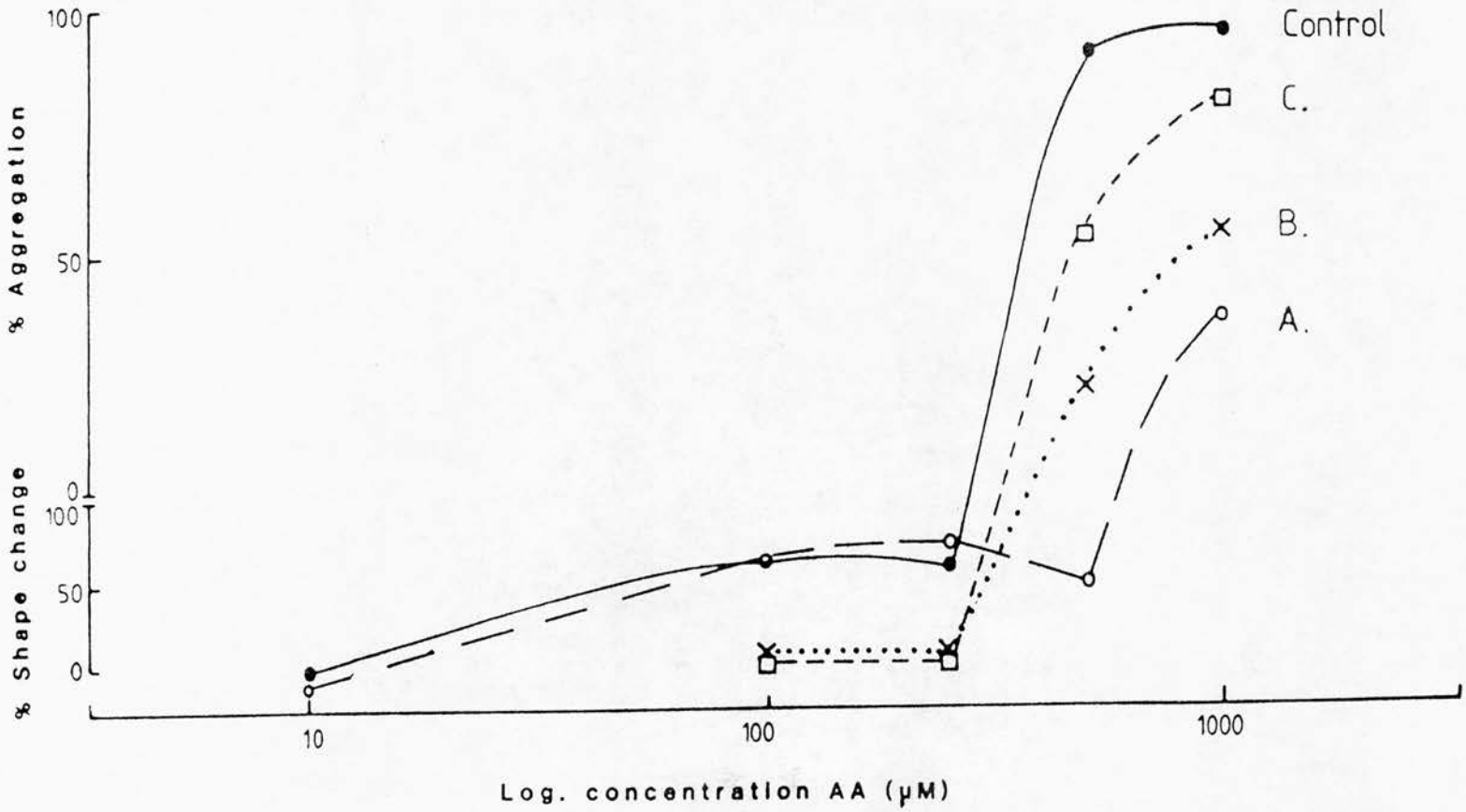


Figure 3.16 The effects of fatty acid derivatives on arachidonic acid responses in citrated rat PRP. Log dose response curves illustrate the AA control curve, (A) C22 : 6 curve and AA curves in the presence of (B) C22 : 4w6 (400μM), (C) C20 : 5w3 (500μM) and (D) C20 : 4Yw6 (500μM).

Figure 3.17 The effect of 5,8,11-ETA on arachidonic acid induced responses in citrated rat PRP. Log dose reponse curves are illustrated for the AA control and in the presence of (A) 5,8,11-ETA (100μM), (B) 5,8,11-ETA (500μM) and (C) 5,8,11-ETA (1mM).


100
50
0
% Aggregation
100
50
0
% Shape change
10
100
1000
Log. concentration AA (μM)
Control
C.
B.
A.

DISCUSSION

It was hoped that studies described in this chapter would help to elucidate the precise mechanisms involved in arachidonate induced activation of rat platelets and possibly clarify the conflicting results reported by other groups. The responses to arachidonate were compared in two platelet systems; citrated rat platelet rich plasma (PRP) and suspensions of washed rat platelets. The results are discussed below.

Citrated Rat Platelet Rich Plasma

Arachidonate added exogenously to rat PRP revealed two types of response dependent upon the arachidonate concentration. Over a range of concentrations, 10-250μM, arachidonate induced ony a shape change response. This effect was fully inhibited by froben, a cyclo-oxygenase inhibitor, EP092, a thromboxane receptor antagonist and only partially inhibited by Dazoxiben, a thromboxane synthetase inhibitor (Fig.3.2). These results would suggest that products of the cyclo-oxygenase pathway are mediators of the shape change response and that the partial inhibition observed in the presence of Dazoxiben can be accounted for by the endoperoxides which can also induce the shape change response. Complete abolition of shape change by the specific thromboxane receptor antagonist would indicate an action at a discrete receptor site.

A comparison of the actions of two stable thromboxane mimetics was made in rat PRP. 11,9-Epoxymethano PGH_2 and EP171 exhibit a

similar profile of activity to arachidonate in rat PRP with only shape change responses being observed. Increasing the concentrations of each mimetic (up to 3μM for 11,9-emPGH$_2$ and 50nM for EP171) did not even produce even a primary wave. EP092, completely abolished the shape change response induced by these mimetics. Froben and Dazoxiben were without any effect. These observations provide a good indication that the effects of these agonists and those of arachidonate are receptor mediated.

Prostaglandin E_1 and Iloprost (stable Prostacyclin mimetic) stimulate prostacyclin receptors to induce an elevation of cyclic AMP and in this instance were found to inhibit shape change induced by arachidonate as well as the two thromboxane mimetics, indicating that the mechanisms underlying the shape change response whether mediated by arachidonate or the thromboxane mimetics are both susceptible to inhibition by elevations in cyclic AMP.

The affinity constants (K_B) for EP092 against 11,9-emPGH$_2$ and EP171 were determined (Table 3.1). These values are in good agreement with the K_B value for EP092 against 11,9-emPGH$_2$ induced shape change in human PRP, suggesting that the mechanism underlying the shape change response in rat PRP is similar to those in human PRP.

A somewhat different effect was observed in PRP when the arachidonate concentration was increased (300-1000μM). What appeared to be an irreversible aggregation wave associated with the release of 5HT was observed (Fig.3.1C-D). This response was

unaffected by both Froben and EP092 demonstrating that arachidonate conversion to prostaglandins and thromboxane is not a prerequisite for this effect. In addition increasing concentrations of the thromboxane mimetics did not elicit an irreversible wave adding support to the above suggestion that the response is not mediated by endoperoxides or thromboxane A_2. Arachidonate itself or products of the lipoxygenase pathway may be the mediators of this response and these possibilities will be discussed later.

The lack of a thromboxane dependent aggregation wave demonstrated in PRP may be due to binding of arachidonate and the thromboxane mimetics to plasma proteins. Albumen has indeed been shown to inhibit arachidonate induced aggregation, and it is possible that it would also inhibit thromboxane mimetic induced aggregation. The addition of increasing concentrations of albumen to a washed platelet system prior to the addition of either thromboxane mimetic or arachidonate would demonstrate whether albumen has an inhibitory effect on mimetic or arachidonate induced aggregation in this system. However, full irreversible aggregation can be induced with thromboxane mimetics in human and rabbit PRP. Since all three species have comparable plasma-protein levels (Chapter 2 - plasma-protein estimation) this would suggest that plasma-protein effects cannot fully explain the lack of aggregation in rat PRP.

Alternatively in PRP preparations the presence of citrate may sequester the calcium in the medium, reducing the extracellular calcium concentrations. In heparinized PRP, the plasma calcium ion concentration is of normal physiological levels (Lages and Weiss,

1981). Similar studies to the ones described here, using heparin as the anti-coagulant would possibly demonstrate whether the presence of citrate in the system was preventing aggregation from occurring.

Washed Rat Platelet Suspensions

When aggregation and the release of 5HT are the biological end points, washed rat platelets exposed to a range of arachidonate concentrations display a bell shaped dose response curve. Peaks of maximal aggregation and release of 5HT occur at approximately 10μM and above 500μM arachidonate (Fig.3.5A & C). Over a small range of arachidonate concentrations (75-100μM) no apparent aggregation was observed (Fig.3.5B). The nature of the three responses were investigated and discussed below.

Low Arachidonate Concentrations (1-50μM)

When washed rat platelet suspensions were stimulated with low arachidonate concentrations, irreversible aggregation waves were observed. The demonstration that EP092 and Froben, completely inhibit these responses provides good evidence to support the notion that rat platelets utilize arachidonate to produce sufficient prostaglandin endoperoxide/thromboxane A_2 to evoke irreversible aggregation mediated through a thromboxane/endoperoxide receptor interaction. Dazoxiben only partially inhibited the irreversible wave indicating that the endoperoxides themselves can induce aggregation. The small inhibition of aggregation which was observed may be explained by the redirected metabolism of prostaglandin H_2 to

prostandin E_2 which has been found to have anti-platelet activity (Bruno et al, 1974). Whether sufficiently high amounts of PGE_2 are produced would need to be investigated. Alternatively, PGH_2 may exert a weaker effect at the thromboxane receptor.

Recently a study by Cerletti and co-workers (1986) demonstrated that the endoperoxide induced aggregation induced response in human PRP was reduced in comparison to that of a thromboxane A_2 and only in the presence of ADP was the extent of endoperoxide induced aggregation similar to that of TXA_2. Whether such an ADP requirement for full endoperoxide induced aggregation occurs in washed rat platelets remains to be investigated. It is possible that the partial inhibition of aggregation evident in the presence of Dazoxiben could be due to a decreased activity of the endoperoxides arising from the absence of ADP in the washed platelet media.

Intermediate Arachidonate Concentrations (75-100μM)

Exposure of washed rat platelets to intermediate arachidonate concentrations (100μM) resulted in a suppression of the aggregatory response which was associated with inhibition of subsequent reactivity to other aggregating agents. The possibility that other prostaglandins metabolised from arachidonate could be causing this effect was investigated since it is known that high concentrations of PGE_2 can exert an anti-aggregatory action on human platelet suspensions (Bruno et al, 1974). It is possibile that PGE_2 produced from arachidonate in rat platelets could be exerting a similar effect.

Pre-incubation of washed rat platelets with Froben should prevent any conversion of arachidonate to its metabolites, including PGE_2 and the subsequent inhibition mediated in the presence of arachidonate would be absent if PGE_2 or other prostaglandins were mediators of the inhibitory effect. However, this was not found to be the case when platelets were pre-incubated with Froben, suggesting cyclo-oxygenase products have no role as inhibitors of the aggregatory response in this instance.

The possibility that either products of the lipoxygenase pathway or arachidonate itself could be exerting this inhibitory effect was examined. Two products of the lipoxygenase pathway 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and its derivative 12-hydroxyeicosatetraenoic acid (12-HETE) have been reported to have an inhibitory effect on platelets, due to suppression of prostaglandin H_2 induced aggregation (Croset and Lagarde, 1983). Their role as possible inhibitors in washed rat platelet suspensions was considered.

None of the four lipoxygenase inhibitors studied were totally specific for the lipoxygenase enzyme. Cyclo-oxygenase activity was inhibited to varying extents demonstrated by the suppression of thromboxane/endoperoxide dependent aggregation induced by low arachidonate concentrations (Table 3.3). However, if the inhibition was mediated by a lipoxygenase pathway product, pre-incubation with a lipoxygenase/cyclo-oxygenase inhibitor should in fact show a reversal of the inhibitory effect of other agents or in fact a reversal of the suppressed aggregation. However, any subsequent response may in

fact be masked if the cyclooxygenase enzyme was also inhibited. It is apparent from Table 3.3 that none of the lipoxygenase inhibitors diminished the inhibition exerted on other aggregating agents. 5,8,11,14-ETYA however at concentrations over 150μM appeared to reverse the suppressed aggregatory response (Fig.3.10) and what looked like an irreversible wave was attained within two minutes. The aggregatory waves observed were not inhibited in the presence of EP092 or Froben, indicating that the response is not mediated by either endoperoxides or thromboxane A_2.

The onset of inhibition was measured as a function of time (Fig.3.12). The inhibitory effect is apparent within 10 seconds. Whether this inhibition is mediated by products of an enzymatic conversion or by another mechanism would need to be investigated since the lag period sufficient to allow activation of enzymes and conversion to inhibitory products is not known and it could be possible that 10 seconds is long enough for this to occur. The inhibitory effect was still evident 15 minutes after exposure to arachidonate, indicating that the mediator of the inhibition was either of a very stable nature or was irreversibly altering the functional integrity of the platelet.

The data so far presented supports the notion that it is the arachidonate itself which is exerting the inhibition but the precise mechanism underlying this anti-aggregatory action has yet to be elucidated.

Modes of arachidonate action

The specificity of the arachidonate-induced inhibition was considered. The results show that ADP, collagen, Thrombin and 11,9-epoxymethanoPGH$_2$ are all inhibited by prior treatment of washed rat platelets with 100μM arachidonate, indicating that the mediator of these effects does not discriminate between various aggregating agents.

It is possible that the inhibition is mediated at the membrane level of the platelet, whereby it could be interacting with the plasma-membrane impairing membrane-linked functions. Collagen and Thrombin are known to accelerate a variety of membrane functions. Collagen stimulates the release of arachidonate from membrane phospholipids and it is the endogenous production of endoperoxides and TXA$_2$ which induce aggregation (Hamberg et al, 1975). Since collagen induced aggregation is inhibited by arachidonate, post-receptor inhibition at one or more sites in the arachidonate pathway is possible. Thrombin induced aggregation on the other hand cannot be accounted for soley by the arachidonate cascade; Lapetina et al (1978) have shown that cyclo-oxygenase and lipoxygenase inhibitors do not inhibit Thrombin induced aggregation yet block the metabolism of arachidonate release from membrane phospholipids. Indeed, Thrombin induced aggregation (low concentrations) was inhibited by intermediate concentration of arachidonate, and since thrombin induced aggregation is thought not to involve the arachidonate pathway, this corroborates the suggestion that the inhibitor acts at a site(s) not involved in the arachidonate cascade.

In these studies, high concentrations of Thrombin (1U/ml) could overcome the inhibition by arachidonate. The precise mechanism underlying aggregation at high Thrombin concentrations is not fully understood but it has been suggested that at high concentrations Thrombin may be acting in a similar manner to calcium ionophores, whereby the membrane permeability is increased. Calcium ionophore induced activation is more difficult to inhibit (Coene et al, 1986).

In addition, 11,9-emPGH$_2$ responses were also inhibited. Since 11,9-emPGH$_2$ activation of platelets is possibly mediated through mobilization of intracellular calcium stores (Brace et al, 1985), occurring independently of arachidonate release from membrane phospholipids, this would support the postulation that the inhibition is mediated at a site other than those at the level of the arachidonate pathway.

It is possible that arachidonate inhibits platelet responses by immobilizing intracellular calcium fluxes. In the studies described here, both the shape change and aggregation responses induced by different agents were inhibited. A recent report (Simpson et al, 1986) has shown that intracellular calcium levels are not elevated in 11,9-emPGH$_2$ induced shape change. Based on these results it would seem unlikely that arachidonate is in fact immobilizing calcium stores since shape change responses induced by 11,9-emPGH$_2$ and other agents were inhibited by arachidonate. In contrast to these findings Brace and co-workers (1985) have reported that arachidonic induced shape change is mediated by increases in intracellular calcium.

The effect of other fatty acid derivatives, linoleic acid and oleic acid were also studied to determine whether these too exhibited similar properties to those of arachidonate.

Linoleic acid is an 18-carbon atom fatty acid with a methylene-interupted double bond system at the 9 and 12 positions. This fatty acid can be converted by various enzyme systems to γ-linoleic acid, dihomo-γ-linoleic acid and arachidonate and so can produce prostaglandins of the 1, 2 and 3 series (Crawford, 1983).

Linoleic, was also found to exert inhibitory effect on other aggregating agents at concentrations in the micromolar range (100μM). What appears to be an irreversible aggregation was exhibited at higher concentrations, the nature of which will be discussed later.

Vallee et al (1980) have shown that Thrombin induced aggregation was inhibited when platelets were incubated with linoleic acid. They attributed this effect to a disruption of prostaglandin and TXA_2 biosynthesis due to an alteration in the phospholipid composition which reduced phospholipase activity. Silver et al (1973) have also demonstrated that linoleic acid inhibited platelet aggregation. However they interpreted this effect to a direct action of linoleic acid on the phospholipase A_2 enzyme. Pace-Asciak & Wolfe (1968) have suggested that the inhibition of aggregation induced by linoleic acid is mediated through irreversible inactivation of the cyclo-oxygenase enzyme. However, inhibition of the cyclo-oxygenase or phospholipase enzymes by linoleic acid cannot explain its anti-platelet activity in these studies because it is known that primary

aggregation induced by ADP and 11,9-emPGH$_2$ occur independently of endogenous arachidonate metabolism (Mills and McFarlane, 1976).

Oleic acid which was also studied is also an 18-carbon atom fatty acid derivative, but with only a single bond at the C-9 position. Mono-saturated fatty acids are not substrates for the oxidising enxymes of the cyclo-oxygenase or lipoxygenase pathway. In these studies low concentrations of oleic acid were found to block ADP induced aggregation (10^{-4} - 10^{-6}M) to a considerable extent. However, at higher oleic acid concentrations, ADP (10^{-4}M) could overcome the block. From these results, it would appear that the inhibition observed with intermediate concentrations of arachidonic acid is not restricted to this fatty acid molecule since linoleic and even oleic acid exhibit similar properties. The question whether all 3 fatty acid derivatives are acting through a common mechanism remains to be elucidated. The demonstration that increasing concentrations of ADP can overcome the oleic acid induced block could illustrate some structure activity specificity in mediating this inhibition, whereby oleic acid is only exerting a weak effect.

From the data presented here, one could postulate that arachidonate and other fatty acid derivates are mediating their effects through perturbation of the membrane. In support of this theory, MacIntyre et al (1984) have demonstrated that cis-unsaturated fatty acid (but not trans isomers) exhibit anti-platelet activity and they attributed these effects to a perturbation of the platelet membranes in specific lipid domains.

Furthermore, studies by Kanaho et al (1983) have shown that certain amphipilic drugs (those having both polar and non-polar structures in the same molecule) of non-related compounds can exert inhibitory effects on arachidonate, collagen and Thrombin induced stimulation of rabbit platelets. They have suggested that such compounds could become incorporated into the lipid bilayer of the platelets whereby bringing about a change in the membrane morphology. Such a membrane perturbation could indirectly affect the microfilaments and as a result prevent pseudopod formation. Microfilaments have been reported to play an important role in the formation of pseudo pods, a prerequisite for the induction of platelet aggregation with some aggregating agents (Pribluda & Rotman, 1982).

High Arachidonate concentrations associated with an irreversible wave and release of 5HT

The irreversible aggregation waves induced with high arachidonate acid concentrations (> 300μM) were not affected by prior treatment with Froben, EP092 or Iloprost, indicative that this effect is not due to a thromboxane/endoperoxide dependent component, even although [^{14}C]-5HT release was associated with these irrversible waves. The lipoxygenase enzyme inhibitors, NDGA and ETYA did not have any effect on these waves. This would exclude the possibility that lipoxygenase products were responsible. In addition, high concentrations of linoleic acid (100μM) also appeared to elicit irreversible waves, which too were associated with [^{14}C]-5HT release. In citrated platelet rich plasma, only shape change was induced at

low arachidonate concentrations but over 300μM irreversible waves were also apparent which were not blocked by Froben or EP092.

These irreversible waves could possibly be explained by a phenomenon known as platelet lysis. Fatty acid molecules may exert non-specific physical effects at the membrane level, causing the platelets and their cellular granules to lyse, resulting in the release of intracellular constituents inducing 5HT. This phenomenon has been reported by Ts'ao and Holly (1979) who demonstrated that high concentrations of arachidonate in artificial media cause platelet membranes to undergo structural changes resulting in lysis. Their observations were supported by electron micrographs illustrating platelet membrane remnants devoid of cellular components. Lysis may also explain the mixed effects observed with the fatty acids studied in platelet rich plasma.

The following fatty acids C22 : 4w6, C20 : 5w3 and C20 : 4W6 (Fig.3.16) at higher concentrations of arachidonate shift the log dose response curve to the left, such that the irreversible wave is evident at a lower arachidonate concentrations. If lysis is the underlying cause of the irreversible wave, an increase in the overall effective free fatty acid concentration due to the presence of these fatty acids in the platelet suspension could cause the lysis phenomenon. An other alternative explanation could be that the fatty acids are preferentially bound to plasma-proteins possibly albumen, resulting in more unbound arachidonate in the media, thus increasing the availability of arachidonate at the platelet membrane.

The other fatty acid studied C22 : 6 induced small primary waves in the absence of arachidonate suggesting that it alone was acting directly on the platelet membrane causing platelet changes. All with the exception of C22 : 4w6 completely inhibited the shape change response induced by low arachidonate concentrations. These fatty acids could be inhibiting the conversion of arachidonate to its metabolites by inhibition of the cyclo-oxygenase enzymes. Indeed C20 : 5W3 and C20 : 4YW6 have been reported to have this activity (Morita et al, 1983A; Ahern & Downing, 1970).

A different profile of activity in rat PRP was observed with the fatty acid derivative C20 : 3w6 (8,11,14-ETA). It only inhibited the shape change at concentrations above 500μM, but in contrast to the other fatty acids studied, higher concentrations of this molecule did not enhance the non-specific irreversible wave but in fact inhibited it to varying degrees (Fig.3.21). It could be postulated that this fatty acid, instead of raising the overall effective free fatty acid concentration, is actually interacting with the arachidonic acid molecule itself, whereby decreasing the amount of free arachidonate available at the platelet membrane. Alternatively C20 : 3w6 may be converted into prostaglandins of the '1' series (Crawford, 1983). The presence therefore of this fatty acid in PRP may not in fact increase the overall effective concentration of free fatty acid but rather fatty acid metabolites may themselves compete with arachidonate for platelet membrane sites.

The evaluation of arachidonates mode of action in rat platelets in this chapter would suggest that the response mediated is very much

dependent upon the concentration of arachidonic acid available to the platelet and other factors in the system such as plasma - proteins and citrate.

At low concentrations, arachidonic acid is taken up by the platelet and the conversion of arachidonate acid to its metabolites, the endoperoxides and TXA_2 is an essential prerequisite for the induction of shape change and irreversible aggregation. It has been demonstrated that the endoperoxide analogue 11,9-emPGH_2 mobilizes intracellular calcium ions in the process of (Brace et al, 1985) and since endoperoxides/TXA_2 production is essential to arachidonate induced responses, this would suggest that both arachidonate acid and mimetics are mediating their effects via a common mechanism, which is likely to involve the mobilization of intracellular calcium.

The finding that arachidonate acid can turn off platelets at intermediate concentrations (75-100μM) whereby they are subsequently refractory to other aggregating agents raises the question as to the physiological status of the inhibited platelets; are they physiologically intact during this period ? In fact, the platelets were found to actively synthesise both cyclic AMP and TXB_2 during the inhibitory period indicating that the platelets are metabolically intact. In addition the platelets were still capable of undergoing aggregation in response to high thrombin concentrations.

Two possible mechanisms were considered by which arachidonate could 'turn off' platelet function; (i) via the formation of inhibitor products or (ii) through the inhibition of critical

enzymes. The results presented here would indicate that products of arachidonate metabolism are not responsible for this inhibition and the antiplatelet activity induced by arachidonate is not due to inhibition of some critical enzymes, but rather that the arachidonate molecule itself is mediating a non-specific effect (also demonstrated by other fatty acids) at the membrane level. The effect may possibly be due to a perturbation of the platelet membrane which renders the platelets refractory to other aggregating agents as discussed earlier. It was noted however, that cyclic AMP levels were increased approximately two-fold over this concentration range of arachidonate acid which exhibited the inhibitory effect. This magnitude of rise in cyclic AMP levels could be adequate to account for the inhibition of platelet function. If this were the explanation, the precise mechanism by which the arachidonate acid was raising cyclic AMP remains to be elucidated. Could the rise in cyclic AMP reflect an effect of arachidonate acid itself on the adenylate cyclase or phosphodiesterase enzyme or could a perturbation of the platelet membrane by arachidonate exert an effect on these enzymes ? These questions remain to be answered.

At concentrations of arachidonate over 500μM the apparent irreversible aggregation associated with release of 5HT, TXB_2 and cyclic AMP production has been postulated to be due to a non-specific (thrombxane-independent) physical effect of arachidonate on the platelet membrane. The fact that TXB_2 and cyclic AMP production occurs at these arachidonate levels indicates that the enzymes systems responsible for their production are intact even though the functional integrity of the platelet as a whole is affected. It is

possible that on increasing arachidonate above a critical threshold concentration, plasma-proteins may become saturated whereby more free fatty acid is available in the surrounding medium. The elevated levels of fatty acids may give rise to the physical effects, including membrane perturbation and activation of platelet enzymes.

When using plasma-free suspensions, the complications of plasma-protein binding are removed and the direct effect of the fatty acid on platelets becomes apparent. The data presented here illustrates how thromboxane/endoperoxide components of arachidonate induced responses may be overlooked in a plasma-protein system and therefore lead to misinterpretated conclusions which may well explain some of the discrepancies seen in other studies concerning the nature of arachidonate mediated effects in rat platelets.

The physiological significance of the inhibition induced by arachidonate whereby it turns off platelet function remains to be established. Linder and Goodman, (1982) has proposed that local concentrations of endogenously released arachidonate attained during platelet stimulation may turn off aggregation when arachidonate concentrations reach a critical level. Thus arachidonate may play a role in regulation of platelet function.

When considering the activation of platelets 'in vivo' the amounts of endoperoxides and thromboxanes produced from arachidonate may well be controlled by the relative levels of free arachidonate as well as other related fatty acids (Bang et al, 1972). In addition albumen itself may be an important controlling factor in haemostasis

since it has been demonstrated that this plasma-protein itself can inhibit platelet aggregation induced by arachidonate, ADP and collagen (Dratewka-Kos et al, 1985). The inhibitory capacity of albumen in circulating blood may vary considerably depending on factors such as the availability of albumen binding sites and the competition for such sites with fatty acids and other substances.

Bills et al (1976) have shown that prostaglandin formation from arachidonate is greater in washed human platelets than in platelet rich plasma suspensions, suggesting that exogenously added arachidonate becomes more available to platelet synthesizing enzymes in plasma-free systems when albumen is absent. This adds further support to the notion that albumen in circulating blood may play an important role in regulating prostaglandin production as well as regulating platelet activation.

Thus the precise mode of arachidonate action may be very different when a comparison is made between 'in vitro' and 'in vivo' situations. Factors such as plasma-proteins (including albumen), the anticoagulant (whether heparin or citrate) and other competing substances (for example other fatty acids) should be considered.

CHAPTER 4

Characterisation of thromboxane receptors

INTRODUCTION

Many compounds exert their biological actions by interacting with specific receptors situated on the plasma membrane of the cell. The more recent isolation and characterisation of receptors has opened the way to a better understanding of the molecular basis of drug actions and the concept has proved to have important practical consequences in the development of drugs for therapeutic use.

A general theory of drug action was first proposed by Clarke who suggested that stimulant drugs occupied specific receptors eliciting a response which depended on the number of these receptors occupied (Clarke 1926, 1929).

This approach was later modified by Gaddum (1937) to consider the action of antagonists which he supposed acted by binding to a receptor without eliciting a response.

In determining the concentration-response relationship for a particular drug, it is usual to express the concentration in logarithmic terms. For agonists these relationships often take the form of a sigmoid curve. A parallel shift to the right of the log dose response curve for an agonist is observed in the presence of an antagonist and this effect was explained by competition between the agonist and antagonist, whereby the agonists competes with the antagonist for the receptor sites.

However, it was demonstrated from experimental evidence that

even when 90-99% of the receptors were being occupied by the antagonist, a maximum response could still be elicited if sufficient agonist was added.

This observation led to the concept of spare receptors, and the suggestion that an agonist need only occupy a small fraction of these receptors to produce a maximal response (Stephenson, 1956) and only in the presence of increasing concentrations of an antagonist will these receptors be occupied. It should be emphasised that these 'spare receptors' are qualitatively similar to non-spare receptors and all are equally available to the agonist.

In competitive antagonism, when an agonist and antagonist are presented simultaneously in solution they are thought to compete for receptors to the exclusion of the others, such that the response is determined by the concentration of the 2 drugs and their relative affinity constants.

In addition to competitive antagonists, 3 other types exist; non-competitive, chemical and physiological antagonists. A non-competitive antagonist acts in an irreversible or nearly irreversible manner and the affinity of such antagonists is so high that the receptor is unavailable for binding by the agonist. Some non-competitive antagonists in fact produce irreversible effects by forming covalent bonds at the receptor site. Chemical antagonism does not actually involve the receptor but in fact another drug which binds to the agonist, inactivating it and consequently preventing the agonist from activating the receptor. The third type of antagonism

is physiological or functional antagonism which involves the action of a drug on another receptor site to the one the agonist binds to, and activation of this other receptor leads to a response which opposes the action of the agonist. An example of this is observed with PGI_2 and TXA_2 receptor agonists, whereby elevations in cyclic AMP opposes the action of thromboxanes. In general, this latter type of antagonism produces effects which are less specific than those of a specific receptor antagonist.

In drug receptor studies, the relationship between receptor activation and response is based upon the assumption that an agonist must occupy the same number of receptors in the presence or absence of an antagonist to produce a given response, and this assumption has led to the use of the dose ratio (the ratio of the concentration of agonist in the presence and absence of an antagonist to produce a given response) to estimate the affinity constant (K_B value) of an antagonist for a particular receptor. The affinity constant may be derived from Schild's equation; $DR-1 = BK_B$ where DR = dose ratio, B = concentration of antagonist, and K_B = affinity constant (Arunlakshana and Schild, 1959).

Determination of the K_B value is one approach used in the classification of drugs according to the receptors on which they act. If a particular antagonist has the same K_B value when measured against different agonists on a given tissue,it is indicative that the agonists are acting on common receptors although not conclusive evidence. Furthermore, comparison of receptors in different tissues which are activated by the same agonist can be made by comparing the

K_B values of a common antagonist. Thus K_B values are generally used as a measure of the affinity of antagonists. On the other hand, equipotent molar ratios (EPMR) can be used as a measure of the effectiveness of different agonists at a given receptor, and this approach allows the relative activities of agonists to be compared on one or more systems. The dose-response relationships for agonists will depend on the density of receptors; low efficacy will tend to result in low potency on sparsely populated systems. Efficacy is the term used to describe the effectiveness of a drug-receptor complex to elicit a response (Stephenson, 1956). Full agonists have an efficacy ranging from zero to an upper limit of one, whilst antagonists have zero efficacy.

The EPMR values are therefore used to compare relative potencies of agonists at a given receptor site and can be used in conjunction with K_B values of antagonists in the classification of receptors. In addition to full agonists which produce a maximal response when occupying only a small percentage of receptors, some compounds (partial agonists), are incapable of producing this maximum even at high concentrations. It is suggested that the efficacy of these compounds is so low that insufficient biological stimulus is generated to ensure a maximum response. At high concentrations, the partial agonist occupies the majority of the receptor pool and hence it opposes the action of a full agonist.

The studies in this chapter make use of the basic concepts of drug-receptor interactions to characterize receptors, and such studies have proved to be a difficult task for the following reasons:

(i) Many pharmacological preparations contain more than one receptor type often giving rise to opposing actions and one prostaglandin may produce both excitatory and inhibitory responses in the same preparation.

(ii) Prostaglandins may trigger the release of other substances which can interfere with the response. These substances may be prostanoid or non-prostanoid in nature.

(iii)Prostaglandins may be metabolised by tissues to varying extents, or if they are highly unstable they may decay spontaneously in neutral aqueous solutions.

It is important therefore, when carrying out differentiation studies to ensure that only one response is being observed and that metabolism and sequestration are limited.

In this chapter, our main concern deals with the thromboxane-sensitive system with the aim of characterizing the thromboxane receptor. Much controversy exists as to whether thromboxane receptor subtypes exist either between tissues or between species.

Earlier reports have suggested that there is a dissociation of thromboxane activity on the vasculature from that on the platelets (Lefer et al, 1980), as well as species differences in the thromboxane receptor on platelets and the vasculature (MacIntyre & Anderson, 1982; Lefer et al, 1983; Mais et al, 1985A and B).

There are limitations when comparing data from different systems in one species as well as between species which should be considered

when critically evaluating data:

(i) When studying isolated tissue preparations, the accessibility of the receptor to agonists and antagonists should be considered since the unionised forms of many antagonists are highly lipophilic and as a result of the distribution between the aqueous and lipid phase in the region of the receptors, the organ bath concentration may not actually represent the true concentration adjacent to the receptor. If the lipid compartment has a high capacity, then a stable situation may arise when the aqueous phase concentration near the receptor is much lower than the bathing fluid concentration.

(ii) Of equal importance is the accessibility of receptors to agonists and antagonists in platelet systems. In platelet rich plasma, the binding of lipophilic agonists and antagonists to plasma-proteins may be a factor which could effectively decrease the free drug concentration, whereby the apparent potency of the drug would be decreased.

(iii)The time that a tissue is exposed to a drug is important since a sufficient period is necessary to allow equilibrium occupancy of the receptor to be attained. This is especially important when studying isolated tissues since many analogues have a slow onset of action whereby they can take up to one hour to reach equilbrium. On the other hand, the platelet system represents a dynamic situation whereby the drug interacts almost instanteously with the receptor, with a rapid onset of equilibrium occupancy.

(iv) Tissue preparations may spend different lengths of time outside the body before an experiment and this factor should be considered since it could possibly affect the sensitivity of the tissue to drugs. Although the time factor may affect the actual potencies of

certain drugs it should not affect the rank order of potency of such compounds.

(v) Finally, the use of non-selective agonists and antagonists could lead to misinterpretation of results, due to their possible actions on other receptor types. In order to make an accurate comparison of one receptor type, it is essential that truly specific agonists or antagonists are used.

Bearing in mind these considerations when comparing data, the following studies involve an evaluation of the thromboxane receptor with regards to its possible heterogeneous nature.

The high instability of both PGH_2 and TXA_2 presents special difficulties in the characterisation of their receptors. One of the first thromboxane mimetics to be synthesized was the endoperoxide analogue 11,9-epoxymethano PGH_2 (Yankee et al, 1976) and because of its selectivity, stability and similar profile of activity to TXA_2 (Malmsten, 1976; Coleman et al, 1981) it is generally used as the standard agonist.

Much work has been carried out in our department on thromboxane-sensitive systems and much progress has been made in this are due to the recent development of highly potent analogues mimicking the action of the parent compound and high affinity competitive antagonists.

In 1975, the discovery that the 16-p-fluorophenoxy $PGF_{2\alpha}$ analogue, ICI79939 had full thromboxane-like agonist activity

prompted the substitution of this aromatic unit into two stable prostaglandin endoperixde analogues, 9,11-ethenoPGH$_2$ and 9,11-ethanoPGH$_2$. The resulting analogues EP011 and EP031 showed high agonist activity on some smooth muscle tissues which was difficult to reverse on removal of the agents from the bathing fluid (Jones et al, 1979). Later studies in which the 16-p-fluoro-phenoxy moiety was introduced into a prostanoid which already exhibited high TX-like agonist activity has given rise to a compound EP171, of outstanding agonist activity (Jones et al, 1985B). More radical chemical alterations of the w-chain of 9,11-etheno and 9.11-ethano PGH$_2$ has resulted in thromboxane receptor blockade. Two examples are the semicarbazone analogue EP045 and the thiosemicarbazone analogue EP092 (Armstrong et al, 1985). Other groups have also synthesized TX receptor antagonists and three compounds which were available to use were ONO11120 (Katsura et al, 1983), BM13177 (Patscheke & Stegmeier, 1984) and AH23848 (Brittain et al, 1985).

The work in this chapter makes use of the wide range of agonists and antagonists available to compare the thromboxane receptor on the platelet from different species, namely man, rabbit and rat. Studies were carried out using plasma-free platelet suspensions to limit the complications which may arise from plasma-protein binding due to the highly lipophilic nature of thromboxane analogues.

RESULTS

Effects of Agonists on human, rabbit and rat platelets

The stable thromboxane mimetic, 11,9-epoxymethano PGH_2 (11,9-emPGH_2) was used as the standard agonist to induce aggregation in platelets throughout this study. 11,9-emPGH_2 has been shown previously to exhibit a similar profile of activity to the naturally occurring compound, thromboxane A_2 (TXA_2) (Coleman et al, 1981), with little agonist activity on PGD, PGE, PGF and PGI receptors.

Washed platelet suspensions from human, rabbit and rat blood were prepared. In both human and rabbit washed platelet suspensions distinct secondary aggregation waves to 11,9-emPGH_2 were not usually observed and more often the primary and secondary waves were kinetically indistinguishable (Fig,4.1A). However, quite often when using rat platelets, a slow onset primary wave aggregation was apparent which merged into a secondary wave of aggregation giving rise to a biphasic pattern (Fig,4.1B). Irreversible aggregation was always apparent at concentrations of 11,9-emPGH_2 over 0.25μM.

Log concentration response curves were established and the concentrations of 11,9-emPGH_2 required to produce a 50% response (the EC_{50} value) in platelets from these species were determined. Six other agonists were studied; 9,11-Azo PGH_2, 9α11α-oxa-10ahomo PGH_2, EP171, EP109, STA_2 and PGH_2. (EP171, EP109 and PGH_2 were synthesised in our laboratories). Their structures are illustrated in Fig.4.2. Each of these compounds behaved as a full agonist and their log

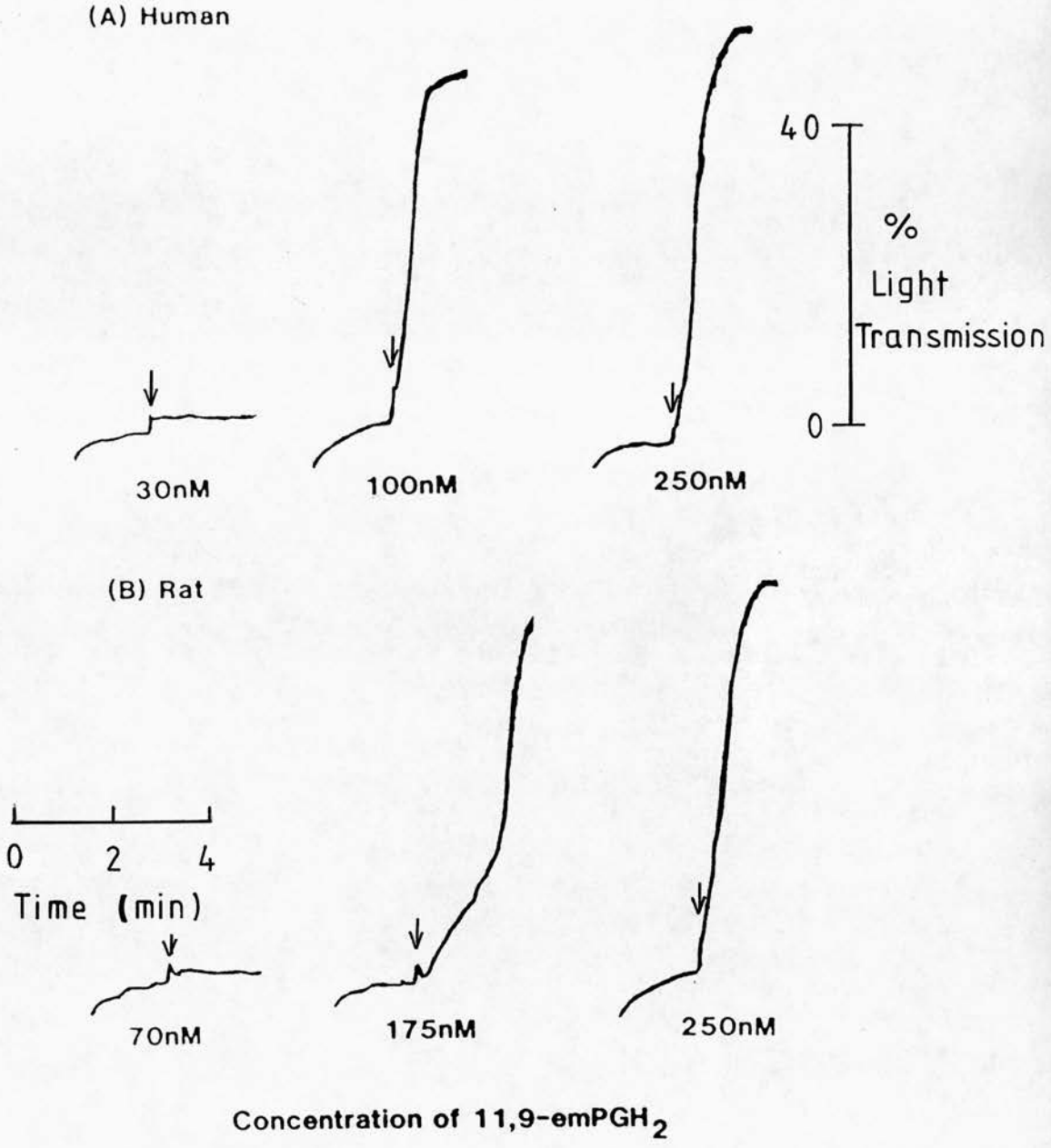
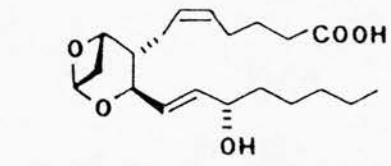
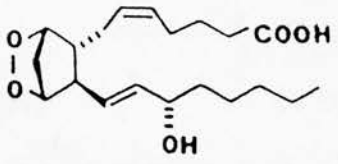


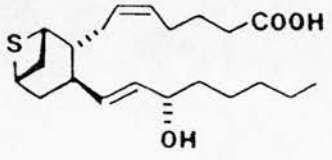
Figure 4.1 11,9-EpoxymethanoPGH$_2$ induced responses in (A) washed human and (B) washed rat platelets.



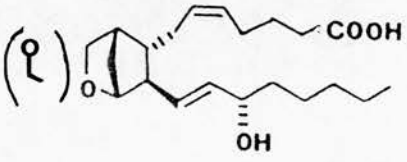
TXA_2



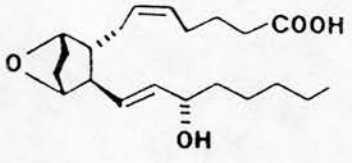
$Prostaglandin H_2$



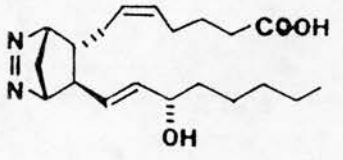
STA_2



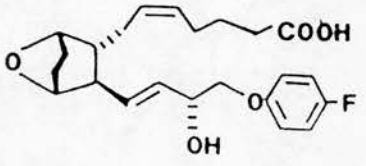
11,9-epoxymethanoPGH_2 (9,11-)



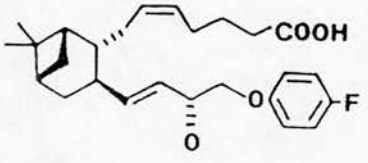
9α,11α-oxa-10a-homoPGH_2



9,11-azoPGH_2



EP171



EP109

Figure 4.2 Structures of the thromboxane mimetics used in this study.

concentration-response curves were parallel to that of the standard agonist. The threshold concentrations for aggregation (TAC) induced by each of the agonists were determined and are shown in Table 4.1. The concentration of each agonist to produce a 50% maximal response was determined and the EC_{50} values are shown in Table 4.2. The equipotent molar ratios of determined from these EC_{50} values and are shown in Table 4.3 (EPMR of 11,9em PGH_2 = 1).

It is evident from these values that EP171 is somewhat more potent than any of the other agonists in all three species, in fact EP171 is the most active thromboxane agonist reported to date. Despite its high potency, the profile of activity of EP171 is similar to that of 11,9-emPGH_2 in platelets from different species (Fig.4.3). It is also evident from these EPMR values that PGH_2, the only natural prostaglandin used in this study, is 1.5 to 2.0 times less potent than 11,9-emPGH_2 in human and rat platelets and equipotent in rabbit platelets. The decreased potency may be due to the redirected metabolism of PGH_2 to other prostaglandins, especially PGD_2 which exhibits anti-platelet activity in some species or possibly due to some decay. However, the log concentration response curves of PGH_2 were found to be parallel to those of the other agonists, indicating that the profile of pharmacological activity of the synthetic analogues closely resembles the profile of the natural compound, PGH_2. Although, these agonists exhibited parallel log dose response curves, STA_2 at high concentrations in human platelets only was found to exert effects different from the other agonists. What appeared to be a reduction in the maximal aggregation response was evident (Fig.4.4). The nature of this effect was investigated.

Table 4.1 Concentrations of Thromboxane Mimetics Eliciting Threshold Aggregation in Washed Platelet Suspensions (nM)

Species	Human	Rat	Rabbit
Mimetic	n = 5	n = 4	n = 4
EP171	1.45 ± 0.05	2.07 ± 0.05	2.02 ± 0.56
STA_2	11.2 ± 2.2	12.9 ± 4.5	5.83 ± 4.5
9,11-azoPGH_2	16.6 ± 4.5	44.8 ± 6.7	11.2 ± 4.5
9α11α-oxoHomoPGH_2	33.6 ± 5.6	78.5 ± 10.1	22.5 ± 1.6
11,9emPGH_2	36.1 ± 4.6	78.3 ± 8.1	48.5 ± 5.2
PGH_2	67.2 ± 16.8	123 ± 22	44.8 ± 16.8
EP109	92.5 ± 6.5	106 ± 5	101 ± 7

(All values are the mean ± s.e. mean)

Table 4.2 EC_{50} Values (nM) for platelet aggregation induced by Thromboxane Agonists

Species	Human	Rat	Rabbit
Tx Mimetic	n = 5	n = 4	n = 4
EP171	2.60 ± 1.10	3.75 ± 0.10	3.60 ± 0.20
STA_2	20.0 ± 4.0	23.1 ± 8.1	10.4 ± 1.0
9,11-azoPGH_2	30.0 ± 9.1	80.0 ± 11.5	20.3 ± 4.0
9α11α-oxoHomoPGH_2	60.1 ± 11.2	140.0 ± 20.0	40.2 ± 3.2
11,9emPGH_2	60.0 ± 4.0	150.0 ± 20.0	60.0 ± 20.0
PGH_2	120.0 ± 30.0	220.0 ± 40.0	80.0 ± 30.0
EP109	165.0 ± 13.0	190.0 ± 10.0	180.0 ± 15.0

(All values are the mean ± s.e. mean)

Table 4.3 EPMR values of Thromboxane Mimetics in Washed Platelet Suspensions (EPMR 11,9-emPGH$_2$ = 1)

(EC_{50} values 65 - 140 nM)

Species	Human	Rat	Rabbit
Tx Mimetic			
EP171	0.041	0.034	0.045
STA_2	0.31	0.17	0.13
9,11-azoPGH$_2$	0.47	0.57	0.24
9α11α-oxoHomoPGH$_2$	0.93	1.00	0.47
11,9emPGH$_2$	1.00	1.00	1.00
PGH_2	1.86	1.57	1.33
EP109	2.50	1.35	2.09

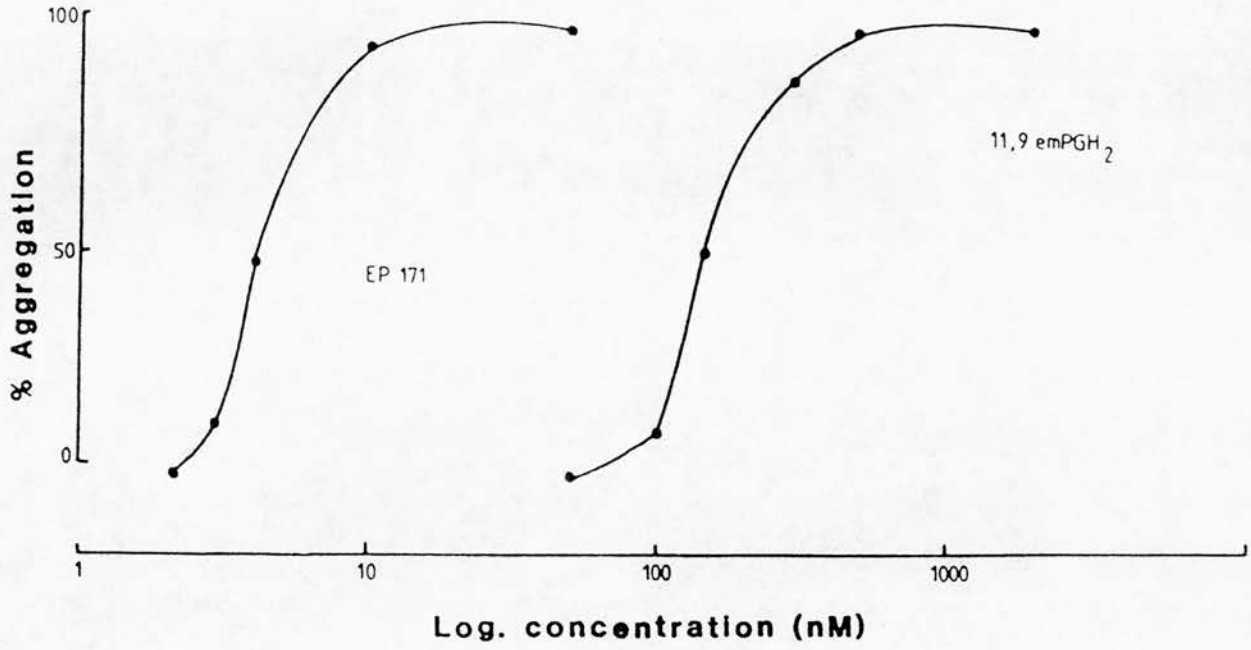


Figure 4.3 Typical log concentration aggregation curves for EP171 and 11,9-epoxymethanoPGH$_2$ on washed human platelets.

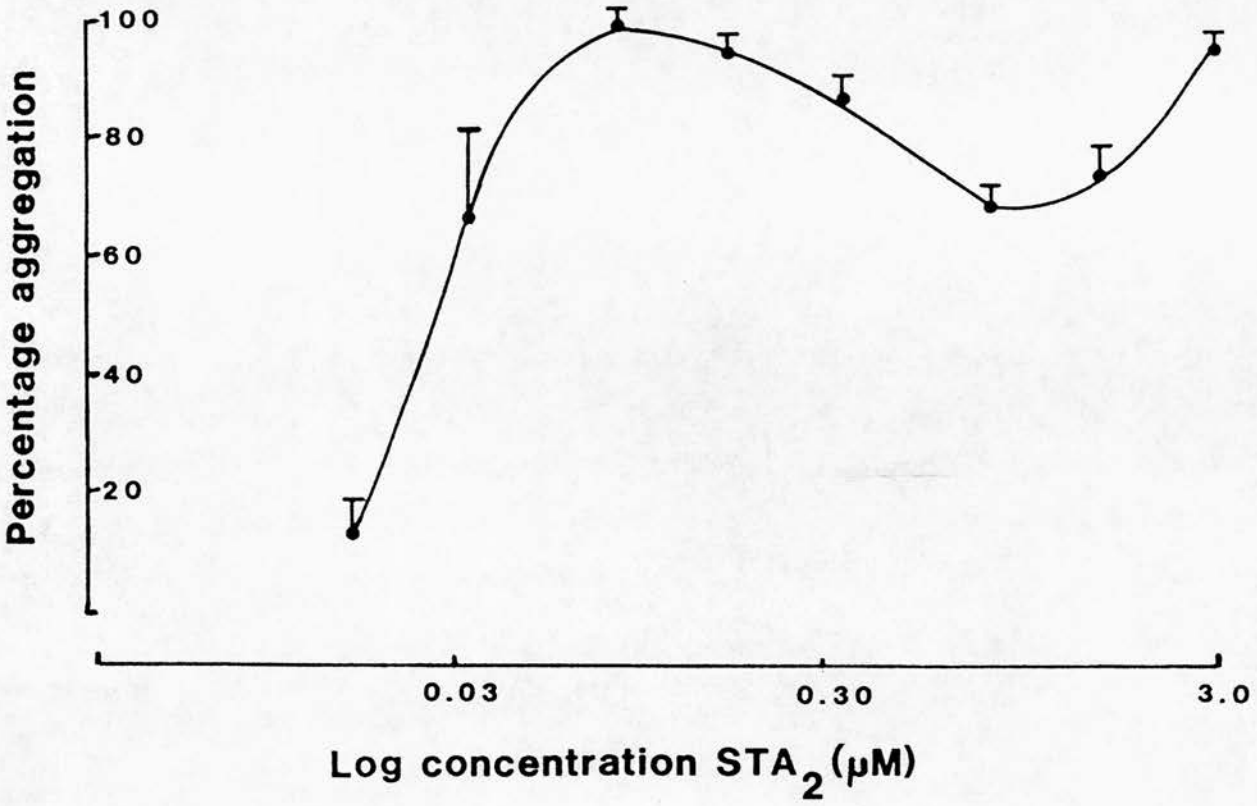


Figure 4.4 Log concentration response curve for STA_2 induced aggregation in washed human platelets.
Each result is the mean and standard error of 4 observations.

PAF induced aggregation (110nM) was studied in human and rabbit platelets in the presence of increasing concentrations of STA_2. Similar experiments were carried out on rat platelets using ADP, as the aggregating agent. The thromboxane receptor antagonist, EP092 was present in all experiments to eliminate any thromboxane agonist-like action. PAF-induced aggregation of human platelets was also tested in the presence of high 11,9-emPGH_2 concentrations. The results are shown in Fig.4.5. STA_2 markedly inhibited the response to PAF on human platelets, but had little inhibitory effect on rabbit platelets. 11,9-emPGH_2 did not inhibit PAF action on human platelets. The ADP response in rat platelets was unaltered in the presence of STA_2 (results not shown).

The possibility that STA_2 was acting on PGD_2 receptors in human platelets thereby elevating cyclic AMP was considered. Using the PGD_2 receptor antagonist AH6809 (Kerry & Lumley, 1985) platelet responses to PAF were observed in the presence of STA_2 and EP092.

A marked inhibition of the control PAF response (110nM) by STA_2 (0.27μM) is illustrated in Fig.4.6(A & B). Pre-incubation with AH6809 (7.5μM) did not reduce the inhibitory effect of STA_2 (Fig.4.6C).

In addition, the effect of the adenylate cyclase inhibitor, SQ22536 on STA_2 inhibition of the PAF response was studied. Pre-incubation with SQ22536 (250μM) was found to have no effect on STA_2 inhibition (Fig.4.6D).

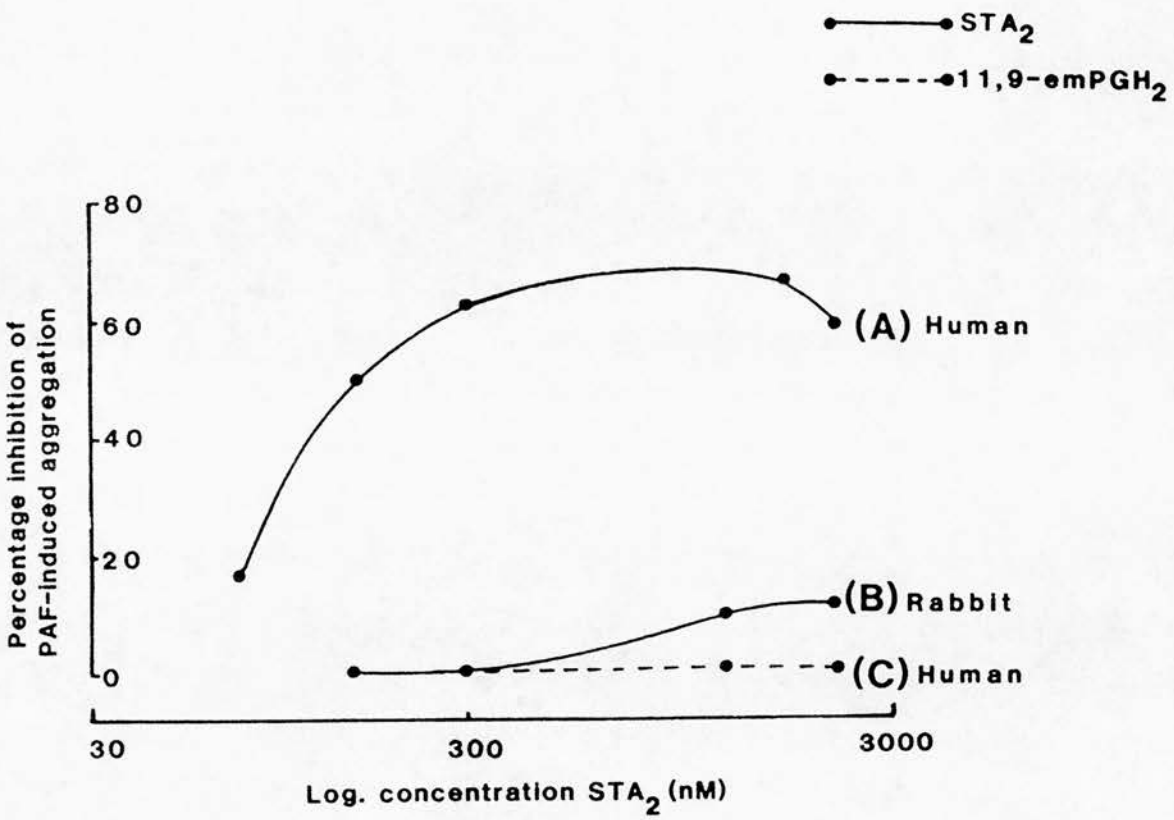


Figure 4.5 Percentage inhibition of PAF (110nM) induced aggregation by STA_2 in (A) human washed platelets, (B) rabbit washed platelets and (C) the effect of 11,9-epoxymethanoPGH$_2$ on PAF induced aggregation in human washed platelets (all experiments were carried out in the presence of EP092 (6μM)).

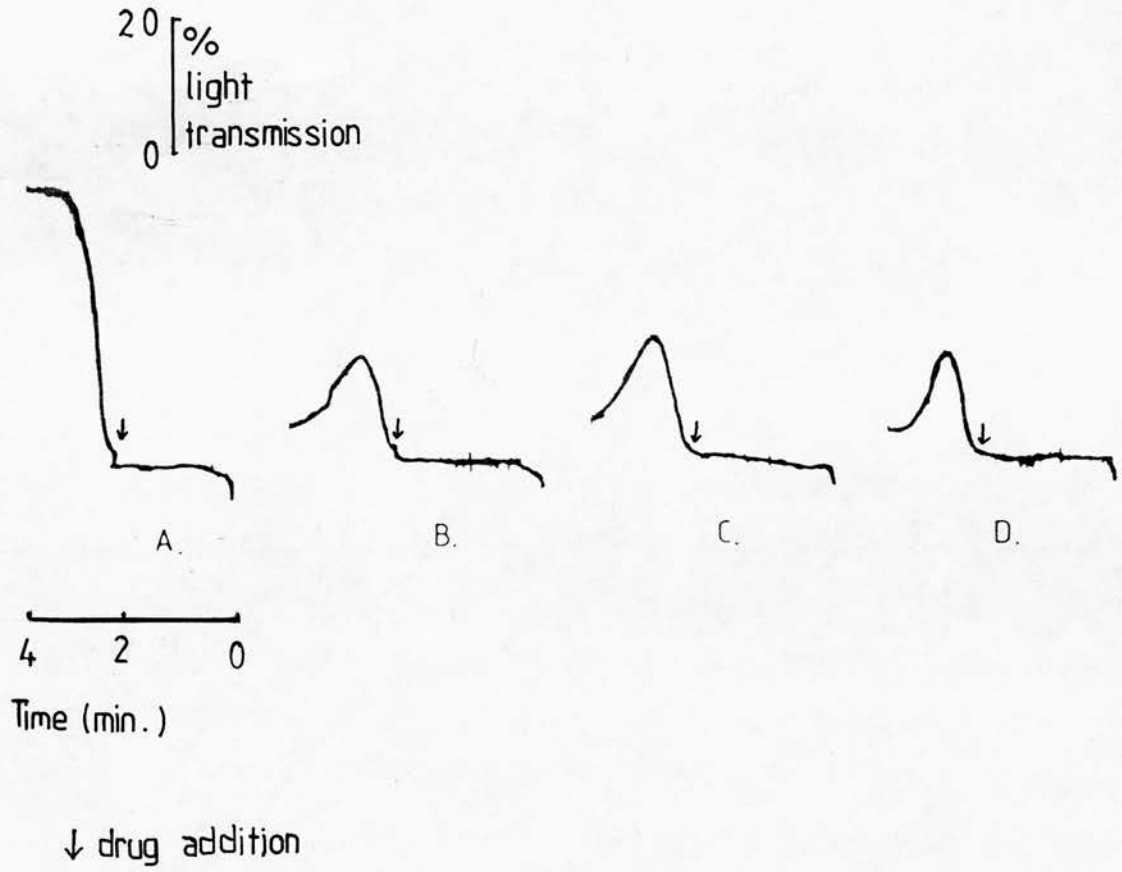


Figure 4.6 The effect of the PGD_2 receptor antagonist AH6809 and the adenylate cyclase inhibitor SQ22536 on STA_2 induced inhibition of PAF aggregation in washed human platelets. (A) PAF control (110nM), (B) STA_2 (0.3μM) and PAF (110nm), (C) AH6809 (7.5μM), STA_2 (0.3μM) and PAF (110nM) and (D) SQ22536 (250μM), STA_2 (0.3μM) and PAF (110nm). (All experiments were carried out in the presence of EP092 (0.7μM). EP092, AH6809 and SQ22536 (added simultaneously) were pre-incubated for 2 minutes prior to addition of STA_2. PAF was added 2 minutes after the addition of STA_2).

Carboxylic-thromboxane A_2 (CTA_2) is structurally very similar to STA_2 and it has previously been shown to produce a primary aggregation wave in washed human platelets but which was rapidly reversed within the 2 minute incubation period. Since CTA_2 has been found to raise cyclic AMP levels, the reversal of primary aggregation could be brought about by the cyclic AMP (Armstrong et al, 1985). It is possible that STA_2 which is structurally very similar, may also have the ability to elevate cyclic AMP. This possibility was assessed by incubating washed platelet suspensions (in calcium free Krebs' solution) with increasing concentrations of STA_2. A similar procedure was carried out using 11,9-emPGH_2 in order to make a comparison with an agonist having no apparent inhibitory effect. Samples were prepared for cyclic AMP binding assay (described in methods chapter). The results are shown in Fig.4.7. An apparent dose dependent increase in cyclic AMP levels is observed.

Partial agonism

The effects of three prostanoids, PTA_2, EP167 and EP204, each known to have partial agonist activity on smooth muscle preparations, were examined on washed platelets from the three species. Structures are shown in Fig.4.8. Shape change and small primary aggregation waves were observed with EP167 and EP204. The log concentration response curves for these analogues were shallower than that of 11,9-emPGH_2 and full aggregation was never attained in platelet suspensions from any of the 3 species. Typical log concentration-response curves for EP204 and EP167 on human platelets are shown in Fig.4.9. Similar responses were observed for EP167 and

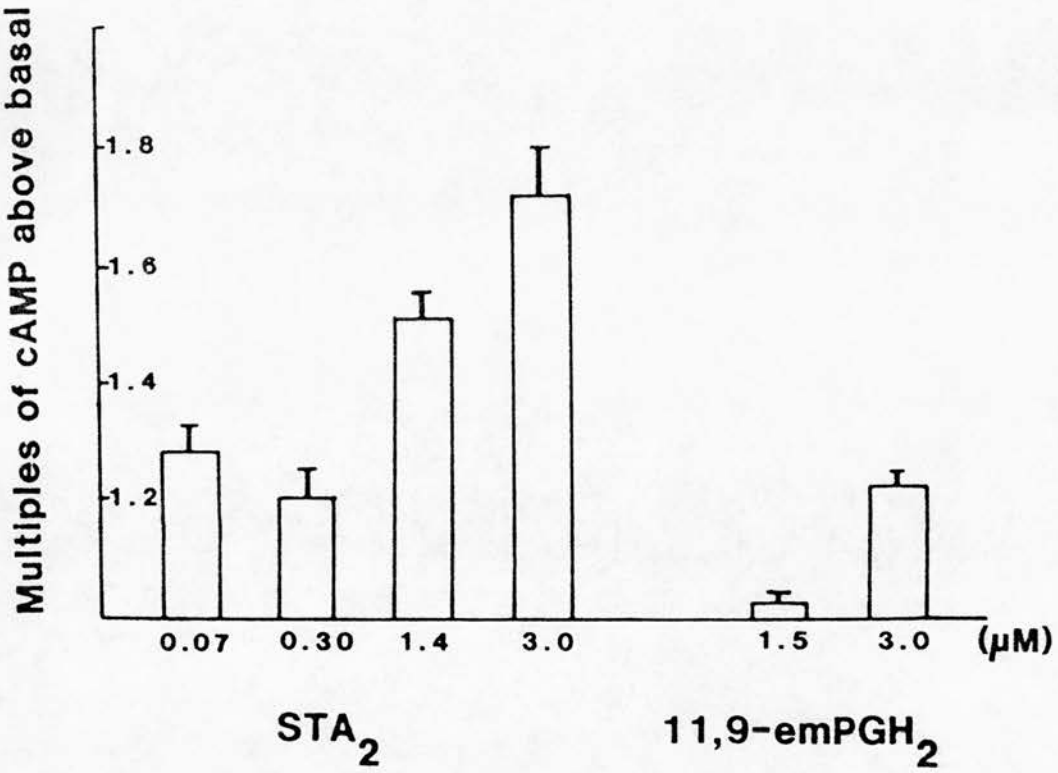
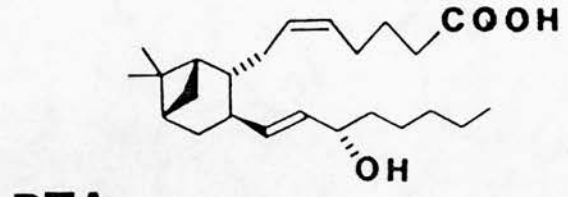
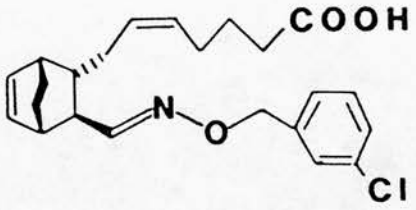


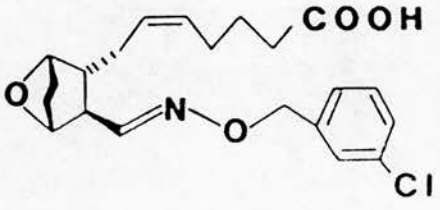
Figure 4.7 Elevations in cyclic AMP induced by STA_2 and 11,9-epoxymethanoPGH_2 in washed human platelets. (Expressed as multiples above basal. Basal cyclic AMP = 1.70 pmol/ml PRP. Each value is the mean ± s.e.m. of 3 experiments (12 samples)).



PTA_2



EP167



EP204

Figure 4.8 Structures of partial agonists used in this study.

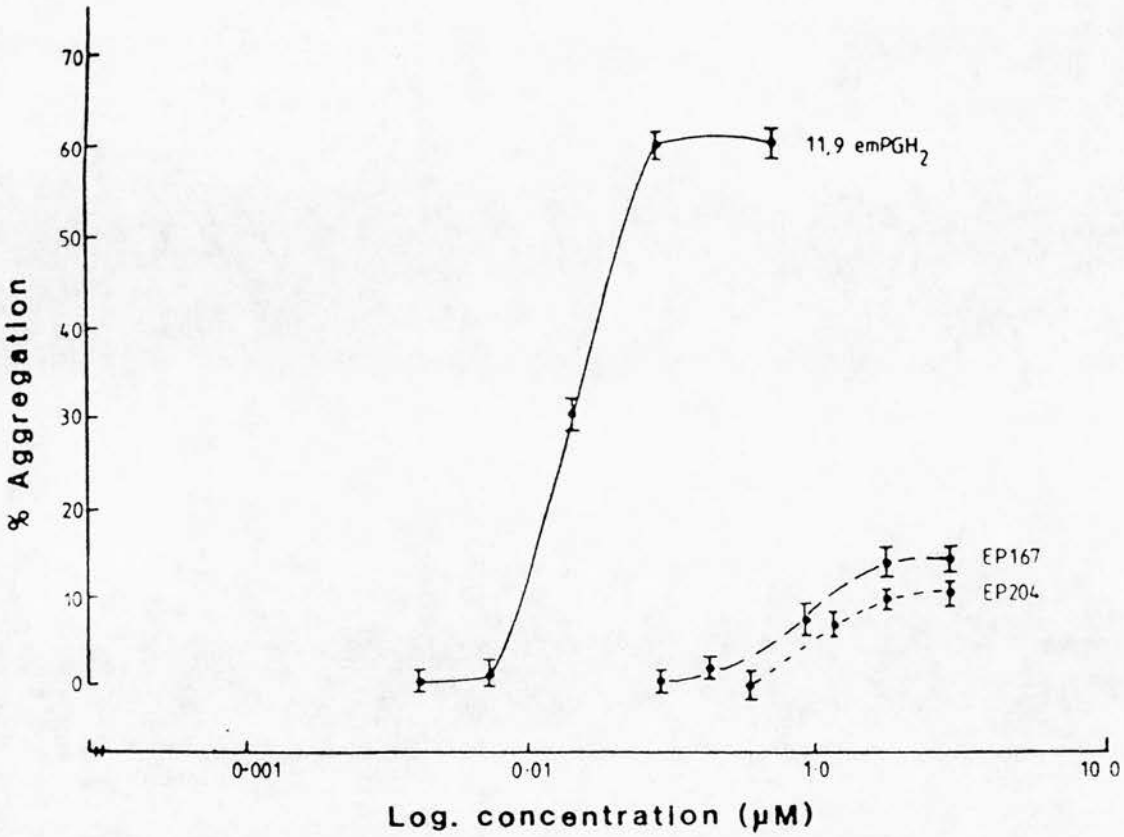


Figure 4.9 Log concentration response curves for 11,9-epoxymethanoPGH$_2$, EP167 and EP204 in washed human platelets.

EP204 in rat and rabbit platelet suspensions. On the other hand, only a small shape change response was observed with PTA_2 in platelet suspensions from human, rat and rabbit, and primary aggregation waves were never observed.

EP171 and EP204

EP167 and EP204 are both structurally similar with respect to the w chain; containing a substituted imino group at the 13 carbon position but with different ring structures. The threshold concentrations of each of these partial agonists required to produce primary aggregation waves are shown in Table 4.4. The ability of these partial agonists to inhibit full aggregation induced by 11,9emPGH$_2$ was assessed; from 5 experiments, the mean concentration of each partial agonist which inhibited the maximal response by 50% (IC_{50}) was determined. These IC_{50} values are shown in Table 4.5. In addition experiments were carried out in which platelet suspensions from the 3 species were pre-incubated with either EP167 (1μM) or EP204 (1μM) before the addition of PAF (110nm in human platelets and 10nM in rabbit platelets) or ADP (1μM in rat platelets) to see whether there was any effect on their aggregatory responses. EP167 and EP204 did not antagonise the aggregatory action of PAF or ADP and in fact a slight potentiation of their responses were observed.

PTA_2

PTA_2 (pinane-thromboxane A_2) has a pinane ring structure with

Table 4.4 Threshold Aggregation Concentrations (T.A.C.) (μM) for the Partial Agonists EP167 and EP204

Partial Agonists	Species		
	Human	Rat	Rabbit
EP167	0.85	1.50	0.55
EP204	1.00	2.85	0.75

(Values are means of 3 experiments)

Table 4.5 IC_{50} values (μM) of EP167 and EP204 and PTA_2 on 11,9emPGH$_2$ induced aggregation

Partial Agonists	Species		
	Human	Rat	Rabbit
EP167	0.64 ± 0.08	0.55 ± 0.07	0.95 ± 0.03
EP204	0.94 ± 0.06	1.11 ± 0.10	0.98 ± 0.06
PTA_2	0.10 ± 0.09	1.25 ± 0.05	11.10 ± 0.05

(Mean ± s.e. mean of 3 experiments)

both natural α and w chains and structurally is similar to TXA_2 (Fig.4.8). In washed platelets from human, rat and rabbit, only very small shape change responses were observed. These effects were inhibited by EP092, indicating that PTA_2 does have some degree of thromboxane-like activity as well as the ability to inhibit aggregation induced by 11,9-emPGH$_2$. The IC_{50} values for PTA_2 against 11,9-emPGH$_2$ are also shown in Table 4.5.

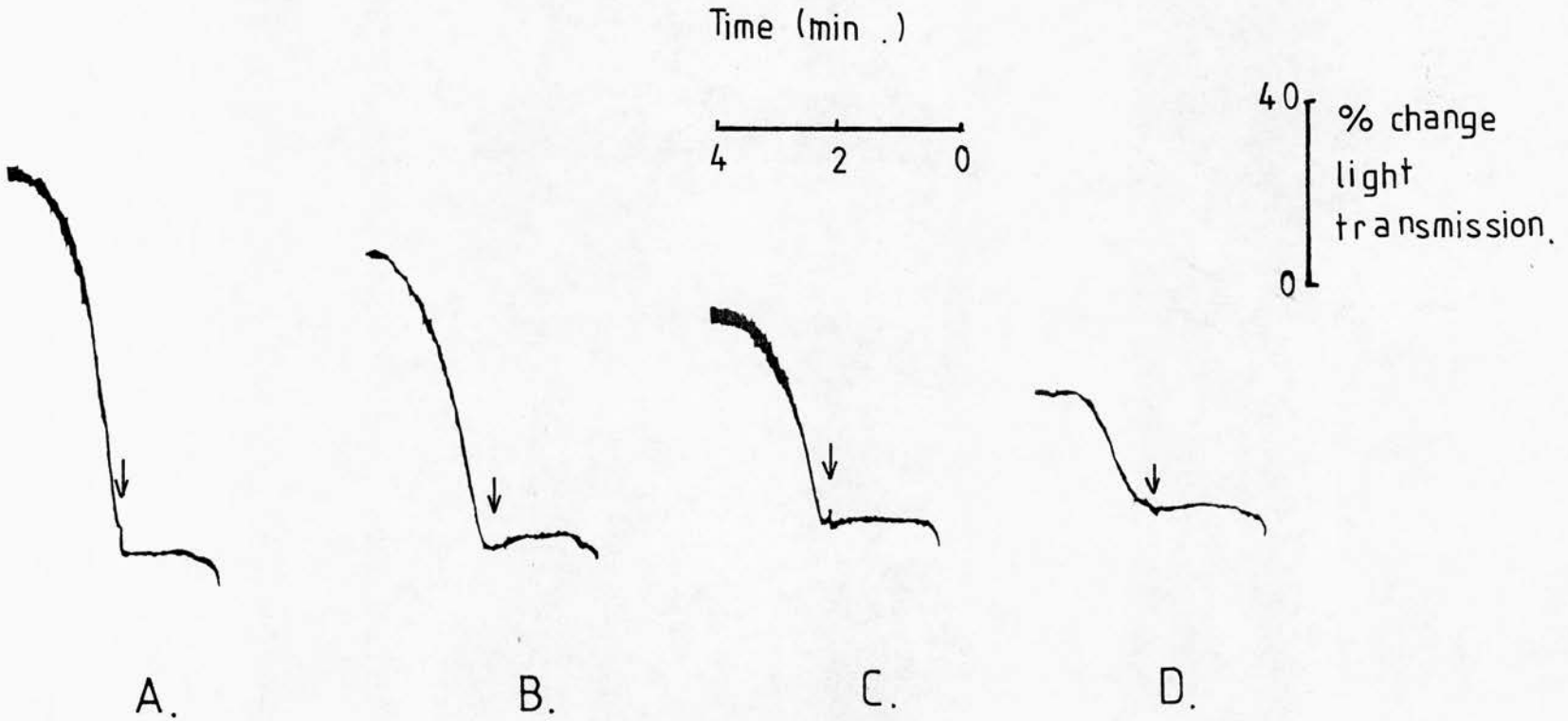
It should also be noted that PTA_2 has been demonstrated to exert dual actions on human platelets (Armstrong et al, 1985) but the precise mechanisms underlying the actions of PTA_2 remain obscure. It has been suggested however that in addition to its thromboxane agonist-like action, PTA_2 in fact exerts an inhibitory effect possibly through the elevation of cyclic AMP and this increase could be mediated through activation of PGD_2 or PGI_2 receptors. Indeed, PTA_2 did reduce the aggregation response induced by PAF in human platelets (Fig.4.10).

The possibility that PTA_2 was exerting similar inhibitory effects in rat and rabbit platelet suspensions was investigated; PAF and ADP induced aggregation in rabbit and rat platelets respectively were tested in the presence of PTA_2. (Froben (10μM) was present in each of these experiments, preventing any endogenous production of thromboxane). Essentially the profile of activity of PAF and ADP in rabbit and rat platelets was unaltered in the presence of PTA_2.

The effect of the adenylate cyclase inhibitor on PTA_2 induced inhibition of 11,9-emPGH$_2$ aggregation (0.3μM) was tested in washed

Figure 4.10 Effect of PTA_2 on PAF induced aggregation in washed human platelets. (A) PAF control curve (110nm), (B) Froben (10μM) and PAF (110nm), (C) Froben (10μM), PTA_2 (2.5μM) and PAF (110nm) and (D) Froben (10μM), PTA_2 (12.5μM) and PAF (110nm). (Froben and PTA_2 were added simultaneously 2 minutes prior to addition of PAF).

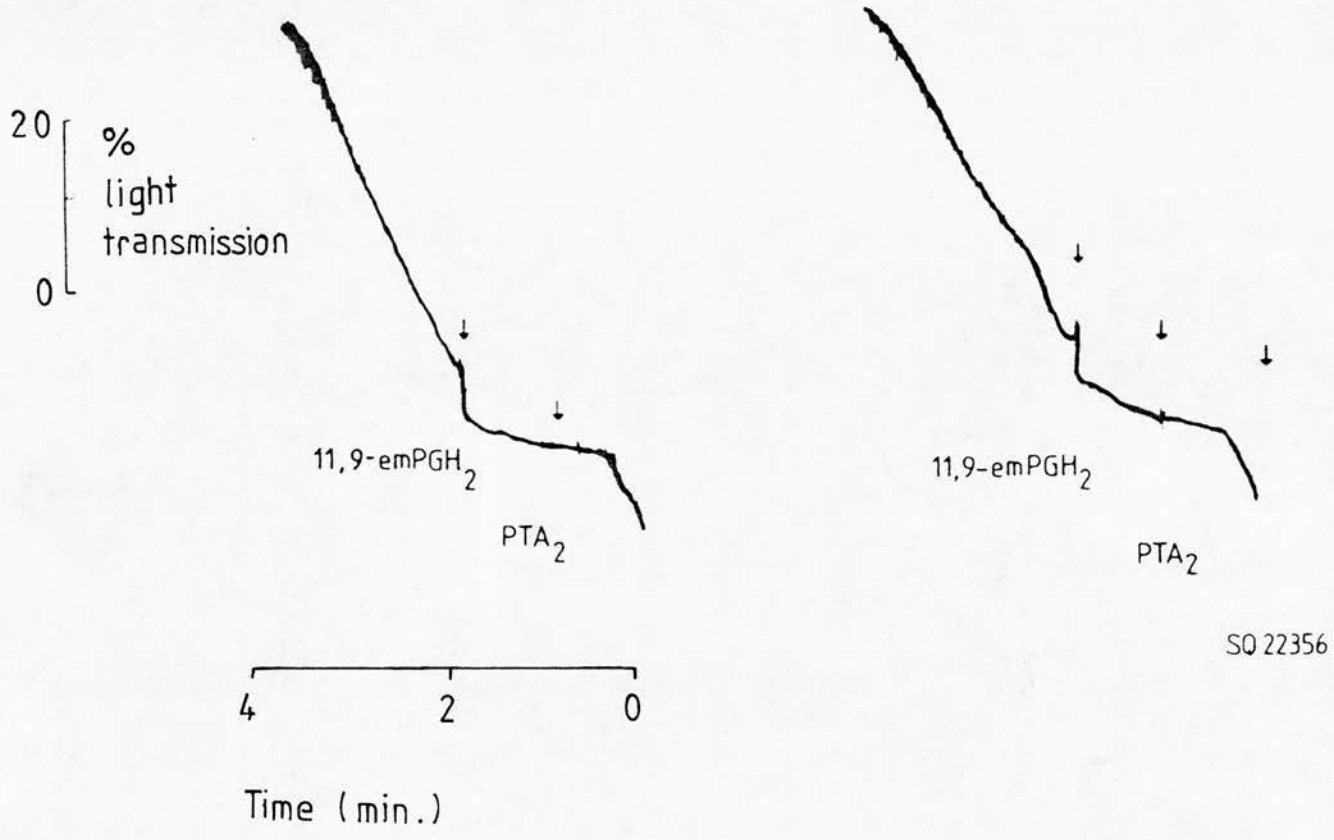
↓ drug addition


Time (min.)
4
2
0
40
% change
light
transmission.
0
A.
B.
C.
D.

human platelets. The IC_{50} concentration of PTA_2 (2.5μM) was tested and in the presence of SQ22536 (250μM) and no reduction in the inhibition of the 11,9-emPGH_2 response was observed (Fig.4.11).

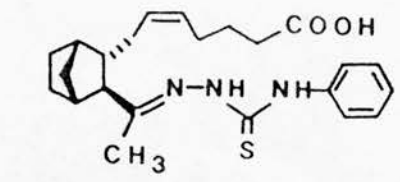
Thromboxane receptor antagonism

Six specific thromboxane receptor antagonists were studied for their ability to inhibit aggregation induced by 11,9-em PGH_2. The structures of these are illustrated in Fig.4.12. In all cases log dose-responses curves to 11,9-emPGH_2 were shifted to the right in a parallel manner. Of the six antagonists EP092 (Armstrong et al, 1985), AH23848 (Brittain et al, 1985), ONO11120 (Katsura et al, 1983) and BM13,177 (Patscheke & Stegmeier, 1984) have been reported to exhibit only specific thromboxane antagonist effects, micromolar concentrations having no effect on cyclic AMP levels. Both EP115 and EP169 (synthesised in our laboratories) were investigated for any PGI_2 or D_2 like activity in platelets from all 3 species. Platelet suspensions were pre-incubated with the antagonists to see whether there was any inhibitory effect on PAF (in human (110nM) and rabbit (10nM) platelets) and ADP (10^{-6}M in rat platelets) induced aggregation, possibly due to elevations in cyclic AMP. Froben (10μM), a cyclo-oxygenase inhibitor was present so as to prevent any endogenous thromboxane synthesis. Neither antagonist appeared to exert an inhibitory effect on the aggregatory responses induced by both PAF and ADP. Cyclic AMP levels in PRP from human platelets following the exposure to EP169 were also measured and it is apparent from Fig.4.13 that there is not a significant increase in cyclic AMP above the basal level.

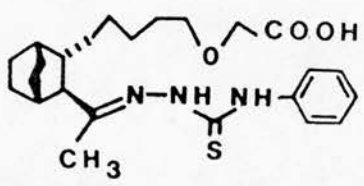


↓ drug addition

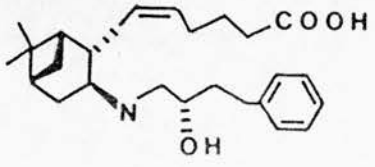
Figure 4.11 The effect of SQ22536 (250μM) on PTA_2 (2.5μM) induced inhibition of 11,9-epoxymethanoPGH_2 aggregation (0.3μM) in washed human platelets.



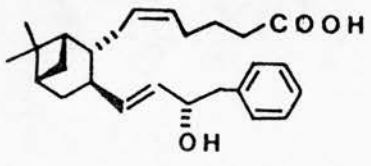
EP092



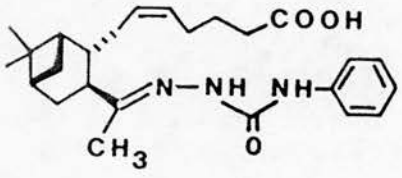
EP169



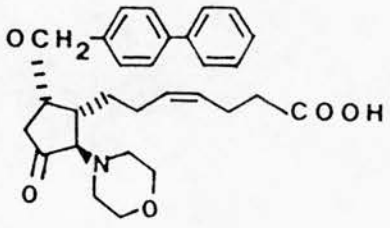
ONO11120



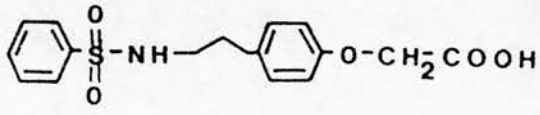
PTA_2 ZII 15(S)



EP115



AH23848



BM13177

Figure 4.12 Structures of thromboxane receptor antagonists used in this study.

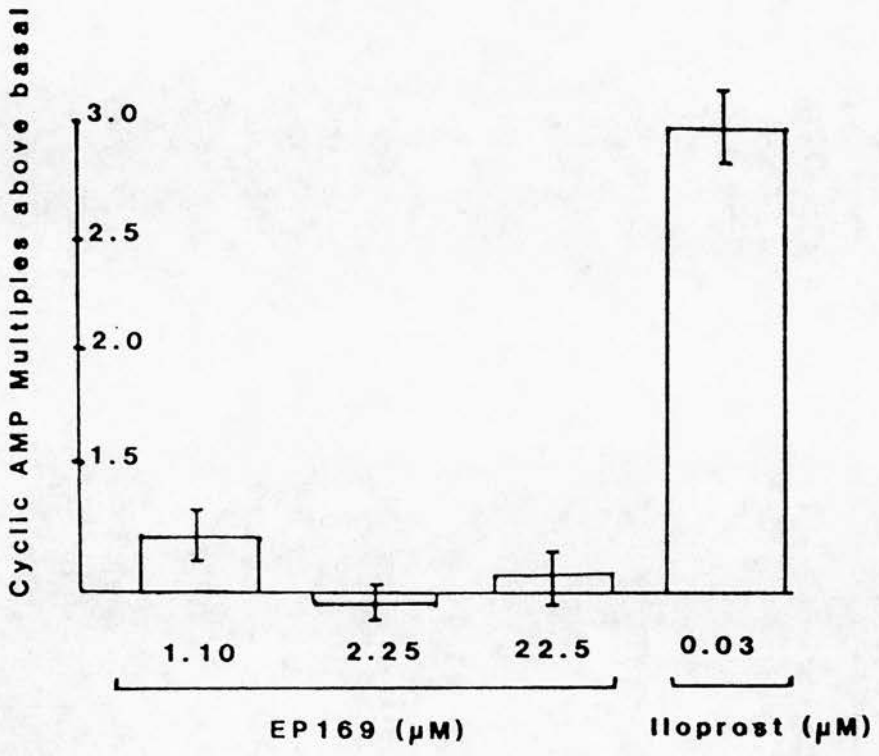


Figure 4.13 The effect of EP169 and Iloprost on cyclic AMP levels in PRP from human blood.
(Values are expressed as the mean ± s.e.m. of 3 experiments (6 samples)).

The aim at this point in the study was to construct Schild plots for each of the antagonists in the 3 species. However, this proved to be practically unfeasible for 2 reasons;

(i) only a limited amount of data can be obtained from one suspension of platelets and so a large number of experiments would be necessary to provide enough data to construct a Schild plot for each of the six antagonists in the three species.

(ii) the nature of aggregatory waves in the presence of the two higher affinity antagonists (EP169 and EP092) in both human and rat platelets differed markedly from that of the control (Fig.4.14). In the presence of both of these antagonists, at a concentration producing a dose ratio of 20 or more, the onset of primary aggregation was slowed relative to that of the control, however irreversible aggregation was still attained within the 2 minutes. This initial slow wave was however not observed in rabbit platelets.

As a result of this when constructing Schild plots the slope tend to deviate from unity at higher concentrations of antagonists. It is possible that these high affinity antagonists (EP169 and EP092) may dissociate more slowly from the receptor, delaying equilibrium occupancy by the agonist, therefore allowing disaggregation to become more effective (Jones et al, 1984). Thus, a compromise was necessary in order to make a comparative analysis of the 6 antagonists. A concentration of each was found which shifted the log dose response curve to the right by 1 log unit, in effect giving a dose ratio of 10, from which pA_{10} values could be determined. (The pA_{10} value is the negative logarithm of the molar concentration of the antagonist). The pA_{10} values for each of the 6 antagonists

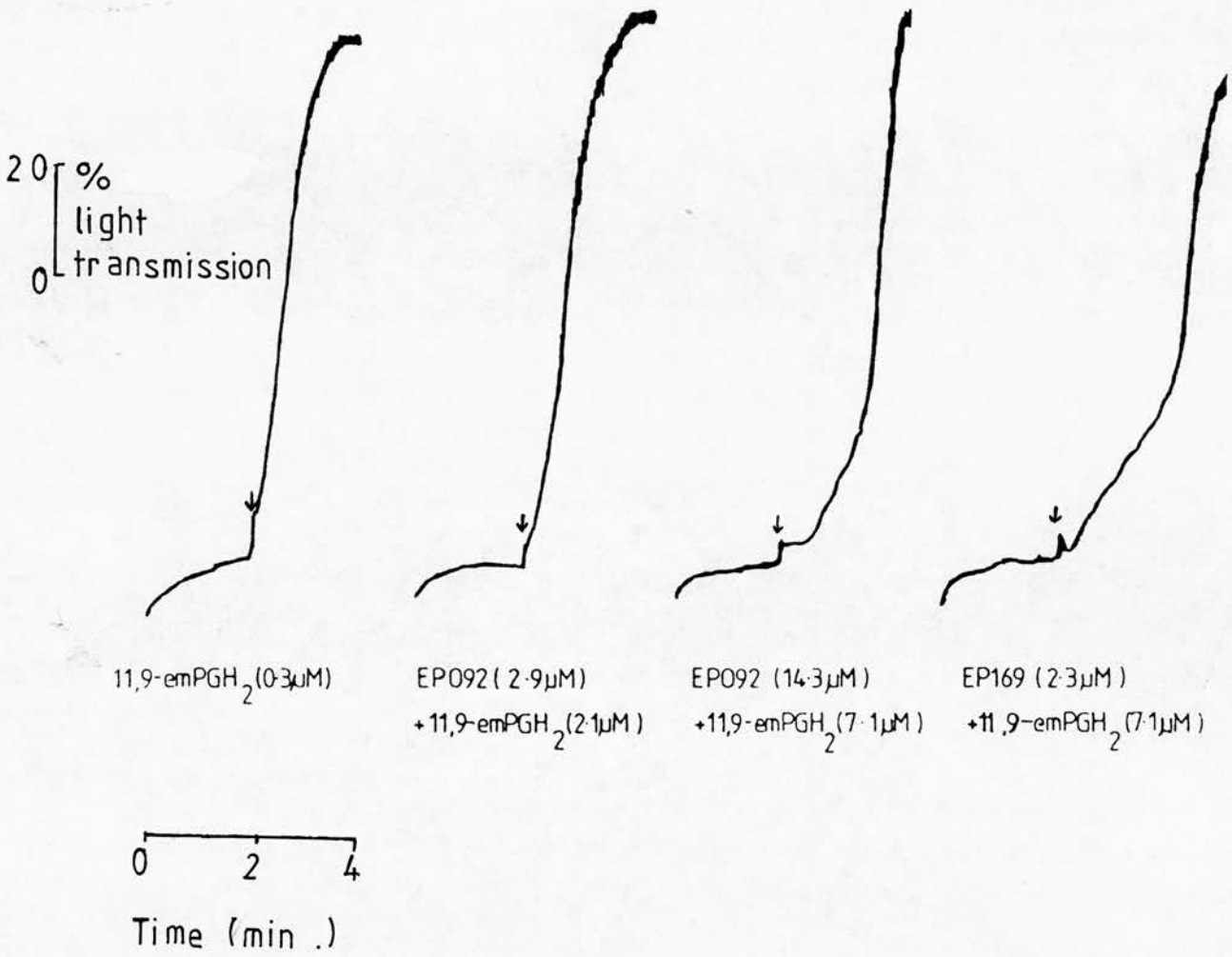


Figure 4.14 The effect of EP092 and EP169 on 11,9-epoxymethanoPGH$_2$ induced aggregation in washed human platelets. (Both EP092 and EP169 were pre-incubated for 2 minutes prior to addition of 11,9-epoxymethanoPGH$_2$).

studied are listed in Table 4.

In these studies, the nature of the block produced by AH23848 differed from the block by other antagonists at concentrations producing a dose ratio of greater than 10. This was only apparent with human and rat platelets and was not observed with rabbit platelets. At concentrations between 0.1-0.4μM in human platelet suspensions and 0.02-0.10μM in rat platelet suspensions, an unsurmountable block was apparent (Fig.4.15). The nature of this effect was investigated by measuring the effect of AH23848 on PAF and ADP induced aggregation in the presence of Froben (10μM). AH23848 (0.1μM) did not appear to have any effect on the primary response produced by PAF (110nM) or ADP (1μM) in human and rat platelets respectively.

A large difference in the pA_{10} values of EP092 and ONO11120 is observed with rabbit platelet but not with human and rat platelets (Table 4.6). The possibility that ONO11120, which has a pinane-ring structure (similar to PTA_2) could have agonist-like activity for the PGI_2/PGD_2 system in human or rat platelets whereby it would exhibit enhanced blocking potency was investigated. PAF induced aggregation in the presence on ONO11120 and Froben was compared to aggregation induced by PAF and Froben alone in both human and rabbit platelet suspensions. No inhibition of the PAF response was observed in either species.

In these experiments the six antagonists were all compared against the standard agonist 11,9-emPGH_2. In addition a comparison

Figure 4.15 The effect of high concentrations of AH23848 on 11,9-epoxymethanoPGH$_2$ log dose reponse curves in washed human and rabbit platelets. Control 11,9-epoxymethanoPGH$_2$ curve in the presence of AH23848 (1.21μM) in rabbit platelet suspensions and AH23848 (0.10μM) in human platelet suspensions.

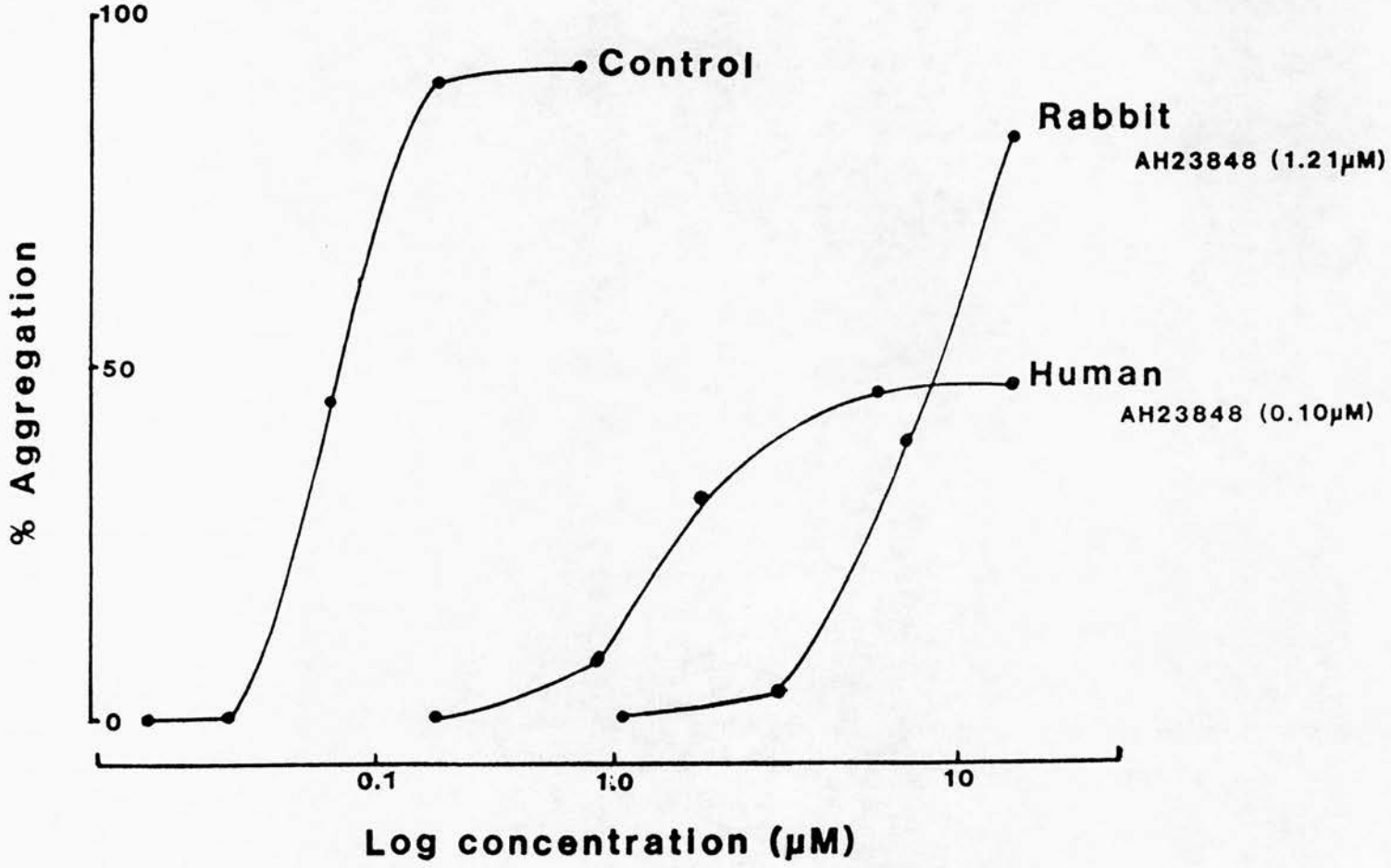

100
50
0
% Aggregation
Control
Rabbit
AH23848 (1.21μM)
Human
AH23848 (0.10μM)
0.1
1.0
10
Log concentration (μM)

Table 4.6 Apparent pA_{10} values for thromboxane antagonism of 11,9-emPGH$_2$ induced aggregation of washed platelets suspensions from man, rat and rabbit

	pA_{10}		
Antagonist	Human n = 5	Rat n = 4	Rabbit n = 4
EP169	7.15 (7.05-7.30)	7.35 (7.30-7.41)	6.28 (6.24-6.32)
AH23848	6.81 (6.72-6.91)	6.96 (6.71-7.26)	5.26 (5.60-5.71)
EP092	6.77 (6.35-6.99)	6.84 (6.69-7.03)	5.75 (5.71-5.89)
ONO11120	6.53 (6.45-6.61)	6.42 (6.39-6.85)	4.99 (4.97-5.00)
PTA⟨⟩ZII 15(S)	6.28 (6.21-6.39)	5.89 (5.81-5.99)	4.74 (4.70-4.82)
EP115	6.02 (5.98-6.07)	6.04 (6.00-6.09)	5.19 (5.11-5.29)
BM13177	5.25 (5.19-5.31)	5.15 (5.12-5.15)	4.91 (4.87-4.95)

of the blocking potency of ONO11120 and EP092 against two other agonists, STA_2 and PGH_2 was made to see whether a similar profile of activity was observed in the three species. As before log dose response, control curves for both agonists were shifted to the right by each of the antagonists and the dose ratio values were converted to pA_{10} values which are shown in Table 4.7.

Effects of TX antagonists on smooth muscle

In addition to the platelet studies, the effects of thromboxane antagonists on 2 smooth muscle preparations were studied, the guinea-pig trachea and rabbit aorta. From these preliminary studies it was hoped to make a comparison of the thromboxane receptors on smooth muscle tissue to those on platelets.

These preparations in terms of thromboxane-like action are relatively 'slow' preparations, whereby the onset of action of the agonist or antagonist is relatively slow (30-60 minutes) and so the only practical method of obtaining complete concentration-response relationships is by cummulative addition of suitable doses to the system.

Cummulative dose responses for 11,9-emPGH_2 induced contraction of the rabbit aorta and guinea-pig trachea are illustrated in Fig.4.16. In the presence of the antagonists (AH23848, ONO11120 and BM13177), the concentration of agonist was suitably increased and from the shift in the log dose response curve the dose ratios and hence the pA_{10} values were determined (Table 4.8).

Table 4.7 PA_{10} values (+ ranges) for ONO11120 and EP092 against 11,9emPGH$_2$, STA$_2$ and PGH$_2$ on human, rat and rabbit platelets

Species	Antagonist	Agonist: 11,9emPGH$_2$	Agonist: STA$_2$	Agonist: PGH$_2$
Human	EP092	6.77 (6.35-6.99)	7.85 (7.82-7.86)	6.61 (6.59-6.76)
	ONO11120	6.40 (6.23-6.52)	6.51 (6.49-6.52)	6.18 (5.96-6.48)
Rat	EP092	6.84 (6.69-7.03)	6.86 (6.69-7.05)	6.58 (6.44-6.80)
	ONO11120	6.35 (6.25-6.65)	6.88 (6.77-6.97)	6.14 (5.93-6.36)
Rabbit	EP092	5.75 (5.71-5.89)	6.04 (5.84-6.21)	6.02 (5.98-6.12)
	ONO11120	5.01 (4.93-5.14)	5.01 (4.92-5.15)	4.42 (4.22-4.73)

Figure 4.16 Typical cumulative dose response curves for 11,9-epoxymethanoPGH$_2$ induced contractions in (A) rabbit aorta and (B) guinea-pig trachea isolated preparations.

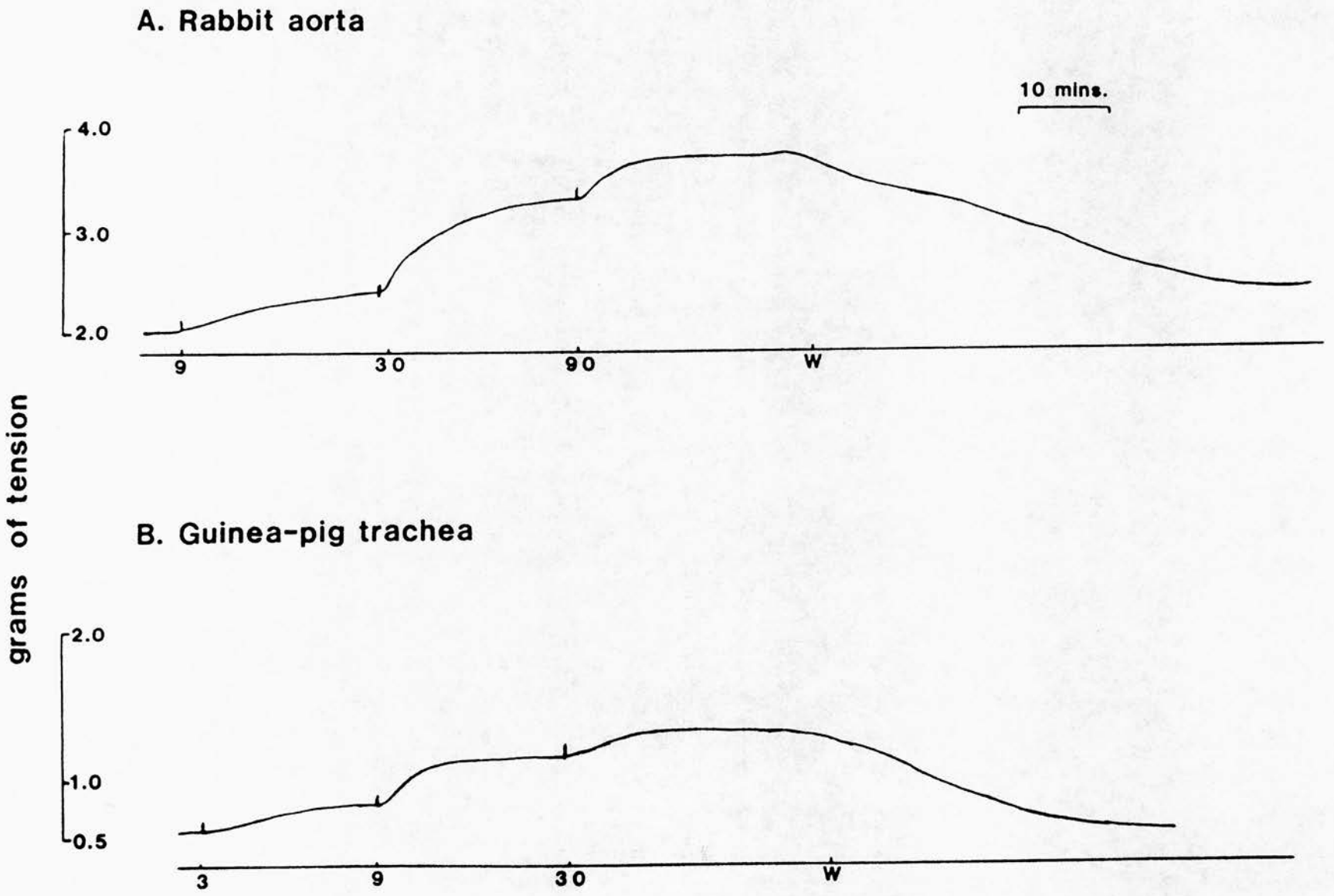

A. Rabbit aorta
10 mins.
4.0
3.0
2.0
9
30
90
W
grams of tension
B. Guinea-pig trachea
2.0
1.0
0.5
3
9
30
W

Table 4.8 pA_{10} values determined for AH23848, ONO11120 and BM13177 on guinea-pig trachea and rabbit aorta isolated preparations. (11,9-emPGH$_2$ was used as the standard agonist)

	Tissue			
	Guinea-pig trachea		Rabbit aorta	
Antagonist		(n)		(n)
AH23848	7.20	1	5.68	1
ONO11120	6.38 (6.16±6.53)	3	4.86 (4.67±4.99)	4
BM13177	5.19 (5.01±5.25)	2	5.09 (4.92±5.22)	3

Ligand binding studies

Preliminary ligand binding studies were carried out on human platelet membranes using the tritiated, 9,11-epoxymethano-PGH_2 ligand. This radioligand was found to exhibit similar biological activity to that of the unlabelled compound on washed human platelets. Separation of bound from free ligand was achieved by rapid filtration of the membranes through microfibre filters. Washed platelet membranes were incubated with increasing concentrations of [^{3}H]-9,11-emPGH_2 (4-400nM) in the presence of a fixed concentration of cold 11,9-emPGH_2 (14μM) for 10 minutes at 37°C. The specific binding of [^{3}H]-9,11-emPGH_2 over this range was determined by subtracting the non-displaceable binding from the total binding (Fig.4.17). It is evident from these curves that on increasing the concentration of [^{3}H]-9,11-emPGH_2 (> 50nM), there is no further increase in displaceable binding (perhaps even a decrease) indicative of a saturable system. It also appears that there is considerable non-displaceable binding which ranges from 50-90% at low ([^{3}H]-9,11-emPGH_2 concentrations and as much as 80-97% at high concentrations. A large degree of non-displaceable binding reduces the accuracy of the assay.

Despite high non-displaceable binding, attempts were made to carry out displacement studies. Fixed concentrations of [^{3}H]-9,11-emPGH_2 (50, 70nM) were incubated simultaneously with the displacing agent, cold, 9,11-emPGH_2 (0-14μM) for 10 minutes at 37°C. The displacement curves are illustrated in Figure 4.18. Unfortunately, the percentage of displaceable binding was low; 25% at 50nM and 30%

Figure 4.17 Binding of [^{3}H]-9,11-epoxymethanoPGH$_2$ to human platelet membranes over a concentration range (0-400nM). Displaceable binding has been estimated as the portion of total binding displaceable by excess cold 11,9-epoxymethanoPGH$_2$ (14μM).
Each result is the mean ± s.e.m. of 3 observations.

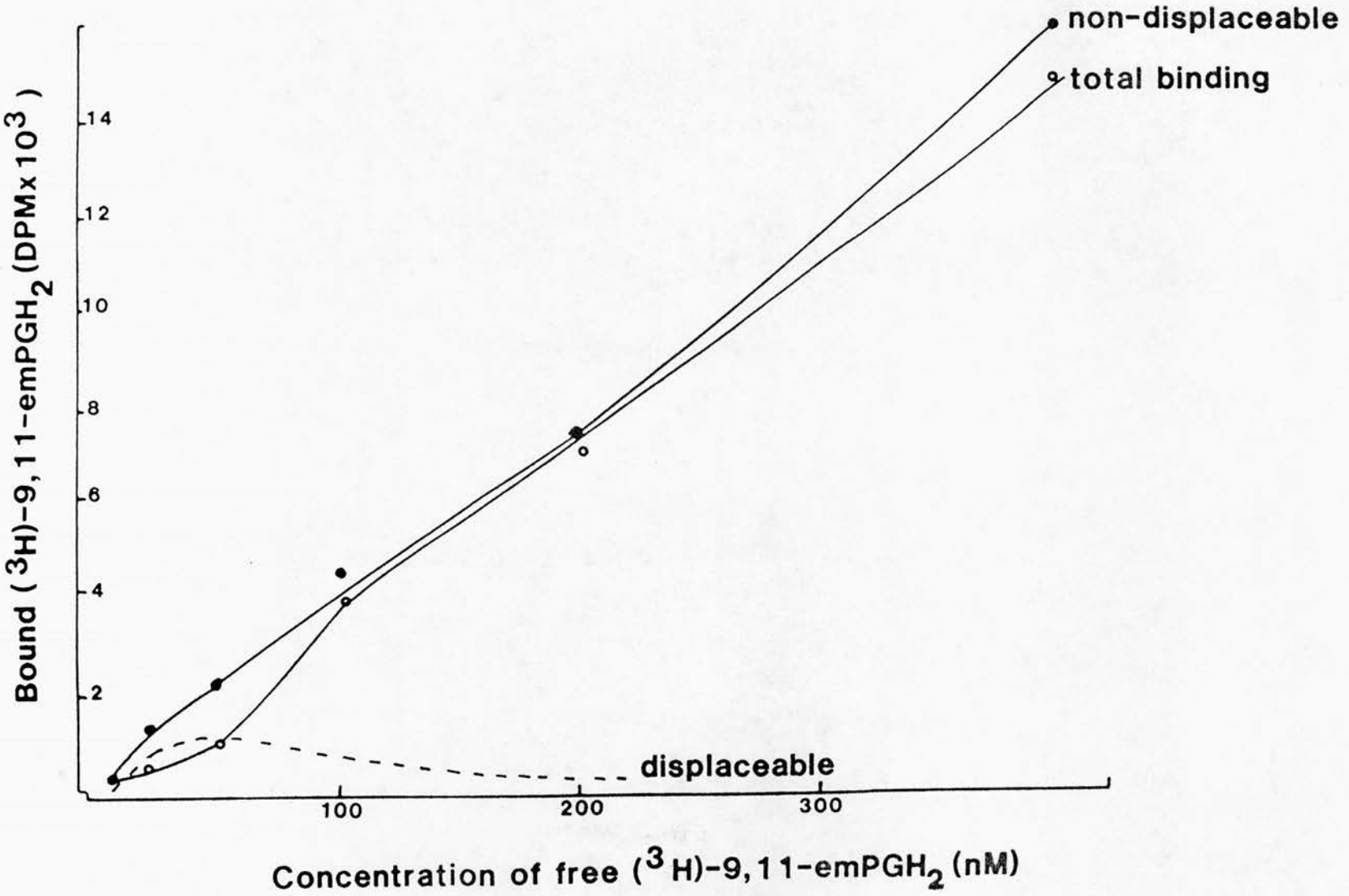
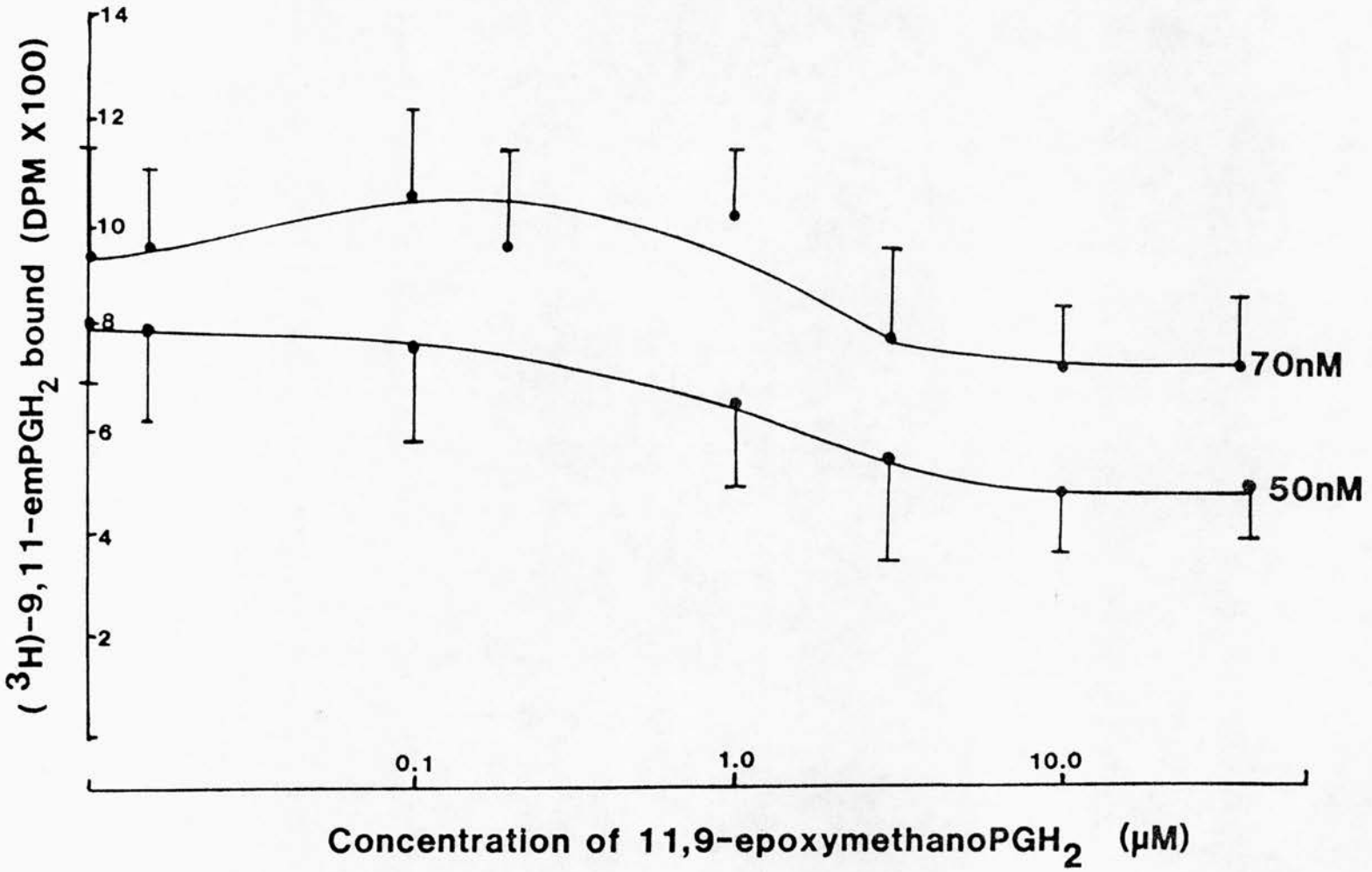

non-displaceable
total binding
displaceable
Bound (^{3}H)-9,11-emPGH$_2$ (DPM x 10^3)
14
12
10
8
6
4
2
100
200
300
Concentration of free (^{3}H)-9,11-emPGH$_2$ (nM)

Figure 4.18 Displacement of [^{3}H]=9,11-epoxymethanoPGH$_2$ (50nM and 70nM) by 11,9-epoxymethanoPGH$_2$.


14
12
10
8
6
4
2
0.1
1.0
10.0
70nM
50nM
(^{3}H)-9,11-emPGH$_2$ bound (DPM X100)
Concentration of 11,9-epoxymethanoPGH$_2$ (μM)

at 70nM. Although a small displacement is evident (1-4μM), the error is large, which makes it difficult to determine the exact concentration range of free ligand which displaces the radioligand. No improvement of the displacement curve was achieved by increasing or decreasing the incubation period, or by increasing the ligand concentration. The underlying cause of the large degree of error is not clear. It may be due to either the low affinity (high dissociation rate) of the ligand or perhaps its high lipophilicity. Whatever the cause, it proved to be unsuitable ligand for extensive radioligand binding studies of platelet membranes.

DISCUSSION

Thromboxane agonist activity

Due to the labile nature of the naturally occurring agents, PGH_2 and TXA_2, agonist ranking measurements are generally carried out using stable synthetic mimetics. The 11,9-epoxymethano analogue of PGH_2 shows a similar profile of activity similar to that of TXA_2 (Coleman et al, 1981) and it was used in these studies as the standard agonist against which the activity of other mimetics were assessed. Seven agonists were tested on the platelet suspensions from the 3 species. It is evident from tables 4.1 and 4.2 that there are not any marked interspecies differences in both the threshold aggregation concentration values and the EC_{50} values.

The apparently higher values observed with rat platelets may be due to the nature of the aggregation wave. The onset of primary aggregation is relatively slow compared to those observed with human and rabbit platelets, but irreversible aggregation is attained within the 2 minute incubation period. As a result, the primary and secondary waves are kinetically distinct giving rise to a biphasic response. It is possible that in rat platelets, this effects is due to a less efficient triggering of the release reaction, and in effect a higher concentration of agonist in necessary to induce release and hence irreversible aggregation.

Table 4.3 lists the EPMR values for the seven agonists studied and it is apparent from this data that the rank order of potency of

the seven agonists is similar in the 3 species. EP171 is the most potent of all the agonists studied and as much as 23 times more potent that the standard agonist 11,9-emPGH$_2$. In fact EP171 is the most potent thromboxane mimetic reported to date (Jones et al, 1985). In platelets from all 3 species, a similar profile of activity of EP171 was observed, and it is evident from Fig.4.3 that the log concentration response curves are parallel in nature, the EP171 curve lying further to the left.

Structure - activity relationships

On analysis of the structure of these agonists, it is evident from Fig.3.2 that all have natural α chains and the 15-(S)-hydroxyl group, despite other modifications to their structures. 11,9-emPGH$_2$ is nominally endoperoxide-like with a modified ring structure - the peroxide oxygen attached to C-9 has been replaced by a methylene group. The 9,11-azo compound is also an endoperoxide analogue which also shows high TX-like activity being about twice as potent as 11,9-emPGH$_2$. The 9α, 11α-oxa-10a-homo analogue may be considered a ring hybrid of PGH$_2$ and TXA$_2$ (Sprague et al, 1983). It has high potency being equiactive with 11,9-emPGH$_2$ on human and rat platelets and slightly more active on rabbit platelets.

The very high activity of EP171 can be correlated with the large increase in potency seen when the 16-p-fluorophenoxy substitution is made on rather weak thromboxane-mimetics (e.g. PGF$_{2\alpha}$ -> ICI79939) (Jones and Marr, 1977). However, it was by no means certain that the substitution of this aromatic unit would produce a marked rise in

activity when the parent was already a potent Tx mimetic.

In EP109 the 16-p-fluorophenoxy substituent has been combined with a pinane ring system. On smooth muscle preparations it behaves as a potent partial agonist (relative maximum responses ranging from 10-30%). On platelets it behaves as a full agonist. This difference may not be of any great significance since the exact nature of partial agonist log concentration-response curves may be dependent on both the receptor density in the system and on the 'gain' of the post-receptor events.

Other agonists which have ring structure related to TXA_2 include STA_2 and CTA_2. STA_2 was in fact used in this study and has proved to be a potent agonist, three to nine times more potent than 11,9-emPGH_2 in washed platelet systems. It might be thought that substitution of sulphur for oxygen at the 9.11 position has maintained thromboxane-like activity. However, the situation is not as simple as this. The synthesis of the 9,11-oxy-11a carba analogue of TXA_2 has been reported in the literature (Sprague et al, 1985) and it is reported to have virtually no Tx-like activity, nor does it block TX-receptors. This situation warrants further investigation in view of opinion expressed by N.H. Wilson (personal communication) that the forcing conditions required for the closure of the 4-membered ring may have resulted in a rearrangement reaction occurring to give an isomeric product. This view is given some credance by the observation that the all-carbon ring version of TXA_2 named CTA_2 is a potent TX-mimetic (Lefer et al, 1980). On human platelet suspension CTA_2 does not produce irreversible aggregation. One

reason for this is its proposed ability to raise cyclic AMP levels (this area will be dealt with later).

The natural endoperoxide prostanoid, PGH_2, appears to be less effective as a TX-agonist (1.3 - 2.0 times less potent than 11,9-emPGH_2) whilst the other agonists which are nominally endoperoxide analogues, 11,9-epoxymethano, 9,11-azo and 9α, 11α-10-oxa-homo PGH_2 were all found to be more potent than PGH_2 itself.

It is probable that the increased potency of the synthetic analogues is due to the fact that modifications to the endoperoxide ring structures introduces chemical stability and therefore decreases the extent to which the molecule will be metabolised. In addition, there may be redirected metabolism of PGH_2 to other prostaglandins which in effect would decrease the amount of PGH_2 available at the thromboxane receptor.

The overall picture arising from these structure activity relationships illustrates that modifcations of the ring structure and the w chain alters the potency of an agonist and without doubt the incorporation of a 16-p-fluorophenoxy group has the most marked effect on potency. Furthermore, it is evident that the presence of the 15-C hydroxyl group is essential for full thromboxane like activity, removal of which gives rise to inhibitory actions. For example, the 15-deoxy-9,11-azoPGH_2 compound has been found to have potent inhibitory action of arachidonic acid and endoperoxide induced aggregation (Gorman et al, 1981).

The data presented here so far illustrates a wide range of structural analogues with varying degrees of aggregatory activity, but despite the difference in relative potencies it should be emphasised that there does not appear to be any marked interspecies differences in their thromboxane-like activities.

A previous study has reported similar data in that the rank order of potency of a range of agonists was similar when compared in both human and rabbit platelet rich plasma (MacIntyre & Anderson, 1982). Based on their results and the data above on the relative agonist potencies it would appear that there is no convincing evidence that thromboxane receptor subtypes exist or in fact that distinct prostaglandin endoperoxide and thromboxane receptors exist. In addition, from a consideration of other published work concerning other thromboxane-sensitive system, including vascular smooth muscle and guinea-pig trachea, there also does not appear to be any marked differences in thromboxane sensitivity with respect to thromboxane/endoperoxide agonists (Jones et al, 1982; Armstrong et al, 1985). Fig.4.19 illustrates the range of EC_{50} values for thromboxane activity in several tissue and platelet systems. A consideration of this data, in conjunction with other studies (Jones et al, 1982; Armstrong et al, 1985) based on relative agonist potencies supports the notion that there are no subtypes of the thromboxane receptor. A consideration of thromboxane receptor sensitivity with respect to antagonists will be discussed later.

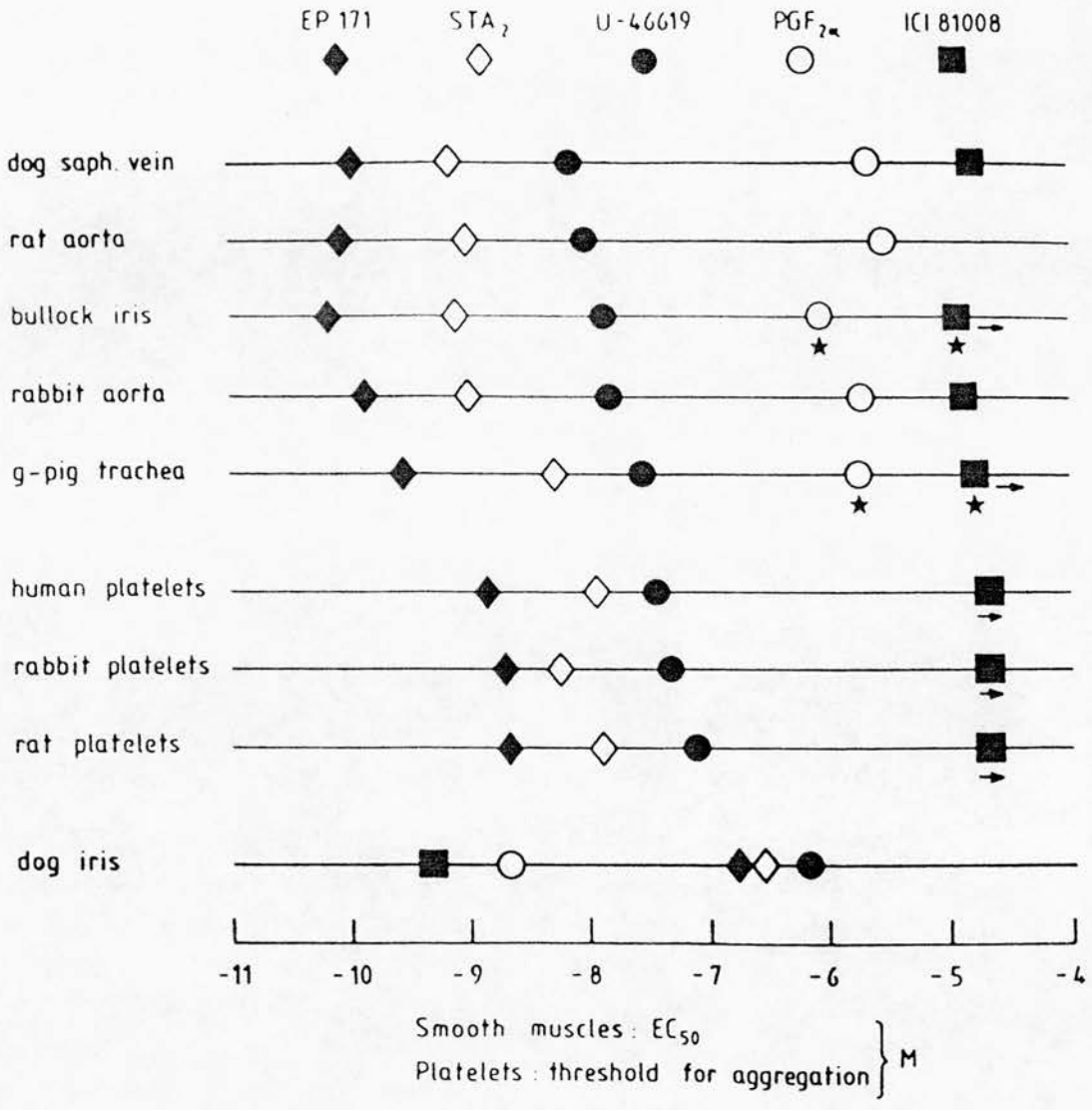


Figure 4.19 Sensitivities of 8 thromboxane-sensitive and 1 PGF-sensitive preparation (dog iris) to selective prostanoids. On smooth muscle preparations the symbols indicate EC_{50} values for contraction whereas on washed platelet suspensions, they represent concentrations producing threshold aggregation waves.

* denotes the presence of 5μM AH6809.

STA_2 activity in platelets

From the data presented here, STA_2 appears to be a potent agonist on platelets from human, rat and rabbit and consistently more active than 11,9-emPGH_2 (3-8 times more potent). These results are in conflict with some recently published data (Narumiya et al, 1986) where the potency of STA_2 was found to be much less (8 fold) in rabbit PRP than when compared to human PRP. A comparison of their EC_{50} values (1.75 and 8.17μM in human and rabbit PRP respectively) with those presented here in washed platelet systems (0.02 and 0.01μM in human and rabbit washed platelets), demonstrate STA_2 to be about 100 fold and 800 fold less potent in PRP from human and rabbit respectively. Although one would expect a decrease in potency of the agonist due to plasma-protein binding, an 800 fold difference is perhaps rather excessive to be explained merely by this.

A comparison of STA_2 activity in PRP and washed platelets was made employing similar experimental conditions (Table 4.9). This data shows only a 10 fold reduction in potency of STA_2 when PRP of rabbit and human blood is used. This magnitude of decreased potency in PRP is what might be expected from plasma-protein binding. Furthermore, the EC_{50} values in human and rabbit washed platelet suspensions were not markedly different in this study.

It would appear therefore that the discrepancies in Narumiya's results on STA_2 activity in rabbit platelets cannot be explained solely by increased plasma-protein binding and as yet the precise reasons remain obscure. Although a full steep dose response curve

Table 4.9 EC_{50} values (nM) of STA_2 induced aggregation in human and rabbit platelets using PRP and washed platelet suspensions

	PRP	Washed Platelet Suspensions
Human	220	20
Rabbit	110	10

for STA_2 was obtained at lower STA_2 concentrations (0.01 - 0.10μM) in all 3 species, a reduction in the maximal response was obtained at STA_2 concentrations over 0.25μM in human platelets. Possible explanations for this effect were sought. Neither the prostaglandin D_2 antagonist, AH6809 nor the adenylate cyclase inhibitor, SQ22536 was found to reverse the inhibition evoked by STA_2. This would suggest that the inhibition is not due to an interaction with a PGD_2 receptor or that elevations in cyclic AMP are solely responsible. However, since SQ22536 does not fully inhibit the adenylate cyclase enzyme, perhaps the degree of inhibition by SQ22536 may not be sufficient to lower cyclic AMP below its threshold inhibitory level. It is well known that only very small increases in cyclic AMP levels are enough to exert full inhibition on platelet activity (Tateson et al, 1977; Harris et al, 1979). The use of a more potent adenylate cyclase inhibitor, a stimulator of phosphodiesterase or ideally a PGI_2 receptor antagonist would perhaps elucidate the mechanism underlying this inhibition. The demonstration that AH6809 did not have any effect on the inhibition would rule out the possibility of a PGD_2 receptor activation of cyclic AMP since this compound is a PGD_2 receptor antagonist and its action is much more direct than enzyme inhibitor. The possibility of STA_2 overcoming the receptor block by AH6809 is improbable since STA_2 is likely to exhibit only a weak action at the PGD_2 receptor reflected by the fact that even at high concentrations, STA_2 never induces full inhibition of PAF aggregation response.

Despite the lack of effect of SQ22536 on the inhibition by PAF, there did appear to be an elevation in cyclic AMP levels above the

basal (by about 1.5-1.8 fold) and although these levels were not high it is possible that they are sufficient to exert the inhibitory effect that was observed. The inhibition appears to be induced only by STA_2 since high concentrations of 11,9-emPGH_2 neither inhibit PAF induced aggregation (Fig.4.5) nor raise cyclic AMP much above basal levels (Fig.4.7).

CTA_2 which is structurally similar to STA_2 has also been found to raise cyclic AMP levels by as much as 4-fold. The onset of the inhibitory effect follows that of the thromboxane-like effect and consequently reversal of the rapidly developing primary wave is seen. Abolition of the cyclic AMP elevating action of CTA_2 may result in irreversible aggregation (i.e. CTA_2 may truly be a full agonist on the thromboxane system). In fact, CTA_2 was studied in the presence of SQ22536 (250μM), but there did not appear to be any reversal of effect. Again the possibility that SQ22536 was not sufficiently inhibiting the adenylate cyclase enzyme to prevent total production of cyclic AMP could possibly be an explanation for this lack of any effect. However, one cannot exclude the possibility that STA_2 and perhaps also CTA_2 may exert an inhibitory effect through mechanisms other than those involving elevations in cyclic AMP.

Partial agonist activity

Three structural analogues all demonstrating partial activity were studied on platelets from man, rat and rabbit.

EP167 and EP204

EP167and EP204 are structurally similar in that they possess identical α and w chains and a bicyclic ring structure. Although full aggregation is never attained with either of these agonist, small primary aggregation waves were obtained as shown in Fig.4.9. Although these partial agonists act on the thromboxane/endoperoxide receptor to induce weak agonist activity it is probable that they do not exhibit high enough efficacy to induce a full response hence the reason why increasing the partial agonist concentration never results in full aggregation. The TAC (Table 4.4) for these 2 partial agonists show that there is not a great difference in the activity when compared in the 3 species. Rabbit platelets appear to be slightly more sensitive to their TX like action. The scatter of values is quite large, whether it is due to the variable presence of a secondary factor or due to small numbers is not known. Increasing the numbers of experiments would show whether or not the slight increased sensitivity of rabbit platelets is a genuine species difference.

EP167 is marginally more potent that EP204 and this increased activity is consistently observed in the 3 species. This increased activity may be attributed to the presence of an etheno group in the ring, since this is the only difference in their 2 structures. When comparing their ability to inhibit 11,9-emPGH$_2$ induced aggregation, EP167 is again marginally more potent (Table 4.6). It is possible that EP167 has a higher affinity than EP204 for the thromboxane/endoperoxide receptor hence the increased inhibitory

effect of EP167. Although in this instance, EP167 is marginally more potent with respect to its agonist activity as well as its ability to inhibit 11,9-emPGH$_2$ aggregation, this need not always be the case when comparing activities of 2 partial agonists since one may have a higher efficacy to produce agonist like action but have lower affinity for the receptor and so therefore will have a weaker inhibitory effect. These two parameters are independent of each other and one way of determining the affinity of these partial agonists would be to carry out ligand binding studies where efficacy is no longer important.

In line with the data from the full agonists, the platelets from the 3 species are equisensitive to these partial agonists, reinforcing the notion that thromboxane receptor heterogeneity is not apparent when looking at agonist like effects.

PTA$_2$

The pinane thromboxane analogue, PTA$_2$ has been found to consistently exhibit partial agonist activity on smooth muscle and human platelet systems (Armstrong et al, 1985). In addition, it has been found to inhibit aggregation induced by non-thromboxane agonists including PAF and ADP. This additional activity may be due to functional antagonism of PTA$_2$ at either PGD$_2$ or PGI$_2$ receptors and may in fact explain the large differences observed in the inhibitory potency of PTA$_2$ on 11,9-emPGH$_2$ induced aggregation observed in human rat and rabbit platelets (Table 4.5).

PTA_2 produced shape change responses, but primary aggregation waves were never observed. These small changes were in fact inhibited by EP092 which would indicate that they are due to a thromboxane agonist like action.

The precise mechanisms underlying the dual action exhibited by PTA_2 on human platelets is not clear, but Armstrong et al (1985) have found that PTA_2 has the ability to elevate cyclic levels above basal and although the increases are small, they may in fact be sufficient to exert an inhibitory effect on platelet aggregation.

Table 4.5 illustrates the wide range in the IC_{50} values for PTA_2 in the 3 species, and it is evident that human platelets are much more sensitve to the blocking action of PTA_2; about 10 fold and 100 fold greater than in rat and rabbit respectively.

The nature of this inhibitory action was investigated in the 3 species by studying the effect of PTA_2 on PAF induced wave aggregation in human and rabbit platelets and ADP induced aggregation in rat platelets. It is apparent from Fig.4.10 that PTA_2 has a significant inhibitory activity on PAF aggregation in human platelets. Negligible inhibition was observed on rat and rabbit platelets. This is compatible with the demonstration that PTA_2 also has the most potent inhibitory effect on 11,9-emPGH_2 induced aggregation in human platelets. In principle partial agonists inhibit full agonist action by occupying the same receptor, but evidence here points to the fact that PTA_2 exhibits additional inhibitory effects. If indeed this inhibitory effect was mediated

by elevations in cyclic AMP through activation of the PGD_2 or PGI_2 receptor then it is possible that the adenylate cyclase inhibitor (SQ22356) would reduce this inhibition through suppression of the enzyme activity. An experiment whereby human platelets were pre-incubated with SQ22356 (250μM) before the addition of PTA_2 was carried out and as illustrated in Fig.4.11, the presence of SQ22356 did not reduce the inhibition by PTA_2 on 11,9-emPGH_2 induced aggregation. This at first would suggest that cyclic AMP has either no or only a minor role in the PTA_2 induced inhibition. In spite of these findings it should be considered (as discussed previously) that this SQ22536 compound does not fully inhibit the adenylate cyclase enzyme and so although there may be a reduced amount of cyclic AMP produced, the reduced level of cyclic AMP may still be sufficent to inhibit the response as only small elevations above basal levels are necessary for responses to be inhibited.

With regard to the wide range of IC_{50} values of PTA_2 obtained from human, rat and rabbit platelets, it may be of relevance here that there is also a wide range of inhibitory activities by the prostacyclin mimetic, Iloprost in the same 3 species. In fact the rank order of potency of Iloprost is such that human platelets are the most sensitive and the rat and rabbit least sensitive to its inhibitory action (Armstrong et al, 1986). It is possible that the differences in the IC_{50} values of PTA_2 in these 3 species, may be accounted for by differences in the sensitivity of the PGI_2 or PGD_2 receptors.

Of the 3 compounds exhibiting partial agonist activity, EP167

and EP204 show no inhibition of aggregation induced by non-thromboxane agonists, with only PTA_2 demonstrating the ability to block PAF induced aggregation in human platelets. It is possible therefore on this basis to distinguish between compounds with true partial agonism at the thromboxane/endoperoxide receptor and those with other actions as well as partial agonist activity. PTA_2 falls into the latter category. Since PTA_2, structurally resembles the parent compound, TXA_2 with natural α and w chains, the very weak agonist action of PTA_2 must undoubtedly arise from substitution of the oxane ring system with carbon atoms, and the addition of the 2 bulky methyl groups at the C-10 position.

Other compounds with similar ring structures include EP109. This compound is a full agonist, whose activity must undoubtedly be attributed to the presence of the 16-p-fluorophenoxy group. ONO11120, EP115 and PTA ⟺ ZII also possess pinane ring structures and these compounds are in fact specific thromboxane/endoperoxide receptor antagonists. Their lack of agonist activity must be due to the loss of the 15-(S) hydroxyl group as well as further modifications to the w chain.

Comparison of the actions of thromboxane receptor antagonists

The basic aim of the following studies was to evaluate thromboxane antagonist activity on platelets from human, rat and rabbit blood. The antagonists shown in Fig.4.12 were studied in washed platelet preparations from these 3 species. All have been reported to have selective thromboxane/endoperoxide antagonist

activity with minimal actions on other prostanoid systems.

A comparison of their antagonist activity in the 3 species was made and Table 4.6 summarizes the results of these antagonists in these 3 species.

Several important findings emerge from these results:

(i) Firstly, all 7 antagonists exert significant antiplatelet effects, extending over a wide range of pA_{10} values (4.91 - 7.15), corresponding to almost 100-fold difference in molar concentrations. Despite their wide range of activity the rank order of potency for the antagonists is similar in the 3 species; human, rat and rabbit.

(ii) Secondly, all of the antagonists with the exception of BM13177 are more potent as blocking agents on human and rat platelets, than on rabbit platelets. The pA_{10} values determined for the rabbit platelet thromboxane/endoperoxide receptor are 1 order of magnitude lower than those determined from either human or at platelets. BM13177 is equiactive on platelets from all 3 species.

(iii)Thirdly, ONO11120 is significantly less potent on the rabbit platelet (by 1.5 orders of magnitude) in comparison to the other antagonists which are only about 1 order of magnitude lower than on human or rat platelets.

The possibility that ONO11120 was exerting some additional inhibitory action on human and rat platelets resulting in a higher blocking effect was considered. Essentially, ONO11120 has a pinane-ring system, similar to that of PTA_2 and since this compound has been found to exhibit inhibitory activity possibly due to elevations in

cyclic AMP, the effect of ONO11120 on PAF and ADP-induced aggregation in human and rat platelets respectively was investigated. However, ONO11120 did not exert an inhibitory effect on these agonists. The apparent difference in the antagonist potency of ONO11120 between human/rat and rabbit, is possibly due to a genuine specific effect of ONO11120 at the thromboxane/endoperoxide receptor.

The AH23848 compound although it appears to behave as a competitive thromboxane antagonist at concentrations shifting the log dose response curve by 1 log unit, at concentrations in excess of these (10-400nM in human platelets and 50-100nM in rat platelets), the curves appear to be no longer surmountable indicating that competitive antagonism no longer exists (Fig.4.15). This effect was not apparent when using AH23848 in rabbit platelets even although much greater concentrations of the compound were needed to produce a block. The primary aggregation waves of PAF and ADP in human and rat platelets respectively were not inhibited in the presence of AH23848. This would suggest that AH23848 is unlikely to be elevating cyclic AMP at these concentrations. A possible explanation for this unsurmountable effect is that AH23848 could itself be acting in an irreversible manner at high concentrations whereby it may be forming covalent bonds at the receptor site. In order to show this, one could incubate a platelet suspension with high concentrations of this antagonist and then finding a suitable method of washing the platelets to remove the antagonists. If the antagonists were binding in a reversible manner the platelets should retain their ability to aggregate.

Another explanation for this effect could arise from the fact that this compound is not very stable, the presence of the hydroxyl group may cause it to readily dehydrate and it is possible at high concentrations, by-products may exert non-specific effects. Despite these findings, it is assumed that AH23848 is behaving as a competitive antagonist at lower concentrations and so the relative potency determined in the 3 species are still valid.

It would appear therefore that with regard to the antagonist potencies, the rabbit platelet thromboxane receptor is the least sensitive to the blocking action of all antagonists reflected by lower pA_{10} values, which reflects a lower affinity of the rabbit thromboxane/endoperoxide receptor.

The possibility exists that the rabbit receptor in fact represents an endoperoxide receptor. If this were the case endoperoxide analogues would be more potent in their actions on endoperoxide receptors than on thromboxane receptors, making it more difficult to block, resulting in apparently lower affinities of the rabbit receptor. With this consideration in mind, the blocking potencies of both EP092 and ONO11120 were compared against the naturally occurring parent compound, PGH_2 and STA_2 (the latter nominaly a thromboxane-like analogue) to determine whether there was any difference found between the 3 species.

Table 4.7 lists the pA_{10} values for ONO11120 and EP092 against STA_2, PGH_2 and 11,9-emPGH_2 in the 3 species. With the exception of STA_2 in the presence of EP092 in human platelets this data is

comparable with the pA_{10} values of the same antagonists determined when 11,9-emPGH$_2$ as the agonist. There is very little difference in the potency of EP092 and ONO11120 against STA_2 and PGH_2. From this data there is no compelling evidence to suggest that the rabbit receptor represents an endoperoxide receptor.

The finding that in human platelets EP092 exerts a much greater blocking potency against STA_2 may in fact be related to the additional inhibitory activity of STA_2. If this were the case, the stimulatory activity of STA_2 would be working against 2 opposing inhibitory actions; (i) the specific antagonist effect of EP092 and (ii) the non-selective inhibitory activity of STA_2 at the higher concentrations, therefore producing a larger shift to the right in the log dose response curve, resulting in a larger pA_{10} value which may not be reflecting the true antagonist activity.

The antagonists studied fall into 3 clearly defined chemical classes; (a) those which are basically modified thromboxane/endoperoxide structures, (EP169, EP092, ONO11120, EP115 and PTA ⟨⟩ ZII (S)), (b) those derived from prostanoid structures although bearing no resemblance to thromboxane/endoperoxides (AH23848) and (c) those which bear no resemblance at all to prostanoids (BM13177). Those in class A can be further divided into two groups; EP169 and EP092 which are bicyclic semicarbazone compounds with similar w chains. EP169 also has a modified α chain. ONO11120, EP115 and PTA ⟨⟩ ZII (S) possess indentical pinane-ring structures and natural α chains, but differ in their w-chains. ONO11120 is an aza-pinane derivative developed from the combination

of 2 classes of antagonists, the aza prostanoids (Le Breton, 1979) and the pinane series (Nicolaou et al, 1979). PTA ⟨⟩ ZII(S) differs from ONO11120, where the NH-group at the carbon 13 position is replaced by a double bond. EP115 on the other hand has a semicarbazone group incorporated into the w chain, structurally similar to the w chain of EP092 and EP169, but where the thiol group is replaced by a carboxyl group. AH23848 which falls into class 2, has nominally a PGD_2 like structure, with a modified w chain and the replacement of a diphenyl substituent at the C-9 hydroxyl group. Finally, BM13177, the only non-prostanoid compound, is in fact an achiral molecule and basically a sulphonamide derivative. Of these antagonists, EP169, EP092 and AH23848 are the more potent reflected by their higher pA_{10} values. The increased potency of EP169 may be attributed to the difference in the ring structure. EP169 has essentially a bicyclo-octane ring system whilst EP092 a bicyclo-heptane ring.

Although a range of antagonist structures have been studied, the limited number does not allow a meaningful structure-activity profile to be drawn up.

The data presented here undoubtedly suggests that the rabbit platelet thromboxane receptor is different with respect to its affinity for several antagonists. At present, it is not possible to conclude that these differences represent subtypes of the platelet thromboxane receptor, but it is possible that if a wider range of antagonists were studied one may be found which is actually more potent at the rabbit platelet thromboxane receptor. From several

lines of evidence it has been suggested that the thromboxane receptors on platelets are in fact different from those on vascular tissue (Lefer et al, 1980; Gorman, 1981; Mais et al, 1985A and B). The platelet represents a complex situation whereby the autocatalytic nature of the platelet creates the problem in that it is difficult to determine whether equilibrium occupancy has been reached and, it is therefore possible that complete aggregation occurs well before equilibrium occupancy it attained. On the other hand, with smooth muscle preparations cummulative dose response curves are obtained and equilibrium is assumed to be reached once the contraction with a given concentration of agonist has plateaued. The differences observed in the activity of agonists or antagonists may in fact reflect differences in the two experimental systems due to the difficulties in establishing equilibrium. Furthermore, drug distribution between the aqueous and lipid phase in the region of the receptor may not be uniform in smooth muscle tissue preparations due to the highly lipophilic nature of the many analogues.

The studies described above on the platelets were extended to consider the effect of some of the antagonists on two smooth muscle preparations: guinea-pig trachea and the rabbit aorta. PA_{10} values for ONO11120, AH23848 and BM13177 were determined for these antagonists and Table 4.8 demonstrates that the blocking potency is much lower on the rabbit aorta. In fact the pA_{10} values found for guinea-pig trachea are very similar to those found on human and rat platelets, whilst the pA_{10} values for the rabbit aorta are comparable to those on the rabbit platelet.

Other groups have demonstrated that of several thromboxane-sensitive smooth muscle preparations, the rabbit aorta is consistently less susceptible to the blocking action of various thromboxane antagonists; EP045 (Jones et al, 1982), EP092 (Jones et al, 1984) and AH23848 (Brittain et al, 1985). Despite the constraints of comparing data from different pharmacological systems it is evident from the data presented here and that of other groups that the affinities of thromboxane receptor antagonists on the smooth muscle preparations; guinea-pig trachea and dog saphenous vein are comparable to those found for human and rat platelets. The affinities for thromboxane receptor antagonists for rabbit platelets are indeed very similar to those found on the rabbit aorta, whereby in both systems, a much lower affinity is observed. It would therefore appear that the thromboxane receptor mediating aggregation in human and rat platelets is similar to the corresponding receptors mediating contractions in vascular and respiratory smooth muscle. Perhaps the differences observed on the rabbit aorta and rabbit platelets represent a subtype of thromboxane receptor confined to that species. It is appreciated that direct binding ligands may lead to the elucidation of thromboxane recptor subtypes fulfilling the definitive receptor criteria.

The use of radioligands for characterisation of the receptors has lagged behind the pharmacological approach mainly because of the unavailability of a suitable radiolabelled ligand. Several ligands have been synthesised and used for radioligand binding studies including [^{3}H]-13-azaprostonoic acid (13-APA) (Le Breton et al, 1979; Hung et al, 1983), [^{3}H]-9,11-epoxymethanoPGH$_2$ (Kattleman et al, 1986;

Armstrong et al, 1983 and Pollock et al, 1984), [^{125}I]-cis-13-APO, a structural analogue of 13-APA (Halushka, et al, 1985) and [^{125}I]-PTA-OH (Mais et al, 1985C; Saussy et al, 1984). The use of these ligands have clarified several important properties of the platelet thromboxane receptor with regards to the specificity of binding and its correlation with biological activities. However there are discrepancies among the results on the studies carried out concerning the number of binding components and density of binding sites. Some of these discrepancies may possibly be merely attributed to procedural differences, for example comparing data from PRP versus washed platelet systems and also intact platelets versus platelet membranes.

Radioligand binding studies using an appropriate radioligand may therefore help to characterise the platelet thromboxane receptor from different species and perhaps clarify whether the lower antagonist affinity of the rabbit receptor demonstrated pharmacologically is correlated with a lower binding-site affinity determined from radioligand binding studies. Such studies may also be of great value when comparing responses that are not only very different in character (contraction versus aggregation) but also when one system is protein-rich (PRP) and another a protein-free bathing solution. Thus if binding studies were carried out on smooth muscle tissue, studies of this kind may help to elucidate the differences that exist between rabbit platelet/aorta thromboxane receptors, with those on guinea-pig trachea and human/rat platelets. Using a tritiated labelled ligand 9,11-emPGH$_2$, preliminary studies on human platelet membranes were carried out in this thesis. These studies

yielded unsatisfactory results from which it was not possible to extrapolate any meaningful data. Displacement curves were associated with a large degree of error and at first this was attributed to the lipophilic nature of 9,11-emPGH$_2$ which could account for the high degree of non-saturable binding. It has since been considered that the low affinity of this ligand for the receptor/binding site with a K_D possibly in the micromolar range would contribute to the large degree of error. In these studies the method of separation of bound from free ligand was by filtration and it is possible that in this instance the time elapsing during this separation period was much greater than the actual half-time for dissociation of bound ligand. This would result in a loss of bound ligand from the system. It has been reported that the time required for separtion of bound from free should be less that one seventh of the half-time of dissociation of bound ligand (Bennet, 1978). In addition, the 9,11-epoxymethanoPGH$_2$ ligand has been shown to interact with the thromboxane synthetase enzyme which it inhibits (Moncada and Vane, 1979). When using membrane preparations, error may arise from binding of the ligand to the enzyme, rather than the receptor site and so it may not be the suitable ligand.

The problems encountered in these preliminary studies would possibly be overcome by using a more specific ligand with higher affinity for the putative receptor, lacking in efficacy and radiolabelled to a high specific activity (an advantage when receptor density is low or if biological material was limited). Indeed the [^{125}I]-PTA-OH ligand meets these criteria and recent studies have shown it to display saturable and specific binding to a putative

thromboxane/endoperoxide receptor (Saussy et al, 1985). Their radioligand binding data correlates with biological data. The use of this ligand may in fact provide the necessary data to further characterise the platelet thromboxane receptor in different species. In, recent studies by Narumiya et al (1986) who have used this ligand to compare the binding activity with the anti-aggregatory activity of ONO11120 in platelets from different species. They found specific binding activity on washed human and dog platelets which correlated well with the biological activity but failed to see specific binding on rabbit platelets. Based on these findings they suggested that there are differences in the ligand binding structures of the platelet receptor in different species. Considering the data reported in this chapter, micromolar concentrations of ONO11120 were necessary to produce thromboxane antagonism in washed platelet suspensions. Since, Narumiya et al (1986) in fact used only nanomolar concentrations of the antagonist in PRP system, it is possible that specific binding would have been revealed if higher concentrations of the ligand were used, especially with rabbit platelet membranes.

In summary, this chapter describes the nature of the thromboxane receptor with respect to its sensitivity to agonists, partial agonists, and antagonists in human, rat and rabbit platelets suspensions. The data presented here provides convincing evidence that platelets from all three species are equisensitive to the thromboxane-like action of several agonists and partial agonists.

These findings are in support with the data of thromboxane-

agonist sensitivity on other tissue preparations (Fig.4.19). It is evident that there is no significant difference in the agonist potency. However, differences in antagonist potency are observed in platelets from these three species, the rabbit platelet receptor of lower affinity. Similarly, a marked difference in the affinity of antagonists (EP092, EP045, AH23848) is found when comparing several tissue preparations, the rabbit aorta having a lower affinity for these antagonists (Jones et al, 1984; Humphrey & Lumley, 1986).

In light of these findings could this data suggest the possibility of either more than one binding site at the endoperoxide/thromboxane receptor or perhaps an interconvertable site? The latter situation may occur if there was one binding site, which could be of two interconvertible forms, an active and an inactive state. Only when the agonist binds to the active state is a response produced. Binding of either agonist or antagonist to the inactive state, produces no effect. It is possible that the antagonist only binds when the receptor is in the latter form and perhaps, in the rabbit, this form has a lower affinity for the antagonist.

Obviously, it would be necessary to investigate the nature of the binding site at the molecular level before one could provide an explanation for the difference in affinity.

CHAPTER 5

Diabetic Studies

INTRODUCTION

Diabetes is a condition which occurs either because of a lack of insulin or because of the presence of factors that oppose the action of insulin. The result of insufficient insulin is an increase in blood glucose concentration (hyperglycaemia) as well as other metabolic abnormalities, notably an increase in ketone bodies in the blood. Although hyperglycaemia is a common factor in different types of diabetes, the underlying causes vary, as does treatment and long-term outlook. The majority of diabetics have 'primary' diabetes which can be of two types (i) insulin-dependent and (ii) non-insulin dependent diabetes.

Diabetes is a disease often associated with clinical complications and patients with longstanding diabetes may develop complications affecting the eyes or kidneys (microvascular complications), nerves or major arteries. Coronary artery disease is commoner in diabetics than in non-diabetics, especially younger women with diabetes of long duration in whom the prevailence of this disorder approaches that of men. This contrasts with the differential prevailence in non-diabetics, where the complaint is much commoner in men.

The cause of microvascular and macrovascular complications is not understood, but diabetes mellitus is frequently found to be associated with abnormal platelet and endothelial function. Abnormal platelet deposition and thrombus formation may explain the increased incidence of atherosclerosis and other microvascular

complications. However, other possible considerations concerning the relation between altered platelet and endothelial function and vascular disease must be taken into account; (i) diabetic vascular disease could infact cause the platelet/endothelial defects; (ii) the abnormalities could be both contributory to and result from the vascular disease and (iii) the two may be totaly unrelated.

Despite other possibilities, most studies are carried out on the basis that altered platelet/endothelial function as a consequence of diabetes may contribute in some way to the diabetic vascular disease. Indeed if this is the case, the following findings would be expected:-

(i) Altered platelet function would be apparent in diabetics before the manifestation of vascular disease. Platelet sensitivity to aggregating agents, production of pro-aggregatory prostaglandins and metabolites, activation of platelets 'in vivo' reflected by β-thromboglobulin levels and perhaps 'in vivo' platelet survival are some of the parameters which may be affected.

(ii) Impaired endothelial function may occur and could be reflected by a decrease in production of prostacyclin (synthesised by endothelial cells), an increase in the von Willebrand factor (endothelial protein) and a decrease in fibrinolysis due to diminished release of plasminogen activation by the endothelium.

(iii)An improvement in altered platelet and/or endothelial function with insulin therapy implying that the defect(s) is related to the insulin-deficient diabetic state.

(iv) A detection of platelet microthrombi at some stage in experimental diabetes.

(v) A diminished rate of progression of diabetic vascular disease with anti-platelet drug therapy.

Hypersensitivity of platelets to aggregating agents is a frequent phenomenon occurring in diabetics. Halushka et al (1977) reported that platelets from diabetics produce more prostaglandin E like material upon exposure to aggregating agents such as ADP, adrenaline, and collagen. More recently it has been found that platelets from diabetics synthesise more thromboxane from arachidonic acid than do platelet from control subjects (Colwell et al, 1983; McDonald et al, 1982). Similar results have been obtained in spontaneously diabetic BB Wistar rats, whose platelets produce more thromboxane in response to ADP collagen or thrombin (Subbiah and Deitermeyer, 1980; Gerrard et al, 1980; Landgraf-Leurs et al, 1982 and McDonald et al, 1982). The origin of the increased prostaglandins and thromboxanes is not clear. It has been suggested that platelets from diabetics either free more arachidonate from membrane phospholipids upon stimulation (Halushka et al, 1977; Gerrard et al, 1980) or convert more arachidonate to metabolites (Colwell et al, 1983). In fact, enhanced secondary phase aggregation of diabetics was found to be corrected by the administration of aspirin, (Sagel et al, 1975). In addition, Morita et al (1983B) have postulated that platelets from diabetics have a higher percentage of arachidonate in membrane phospholipids. These findings were in fact demonstrated in diabetics who already had signs of vascular disease, but increased prostaglandin and thromboxane production (Stuart et al, 1979; Butkus et al, 1980) as well as increased platelet sensitivity (Sagel et al, 1975; Halushka et al,

1981; Janka & Demel, 1981) have been reported in diabetics free from microvascular complications (an absence of retinopathy by opthalmologic examination in patients is usually a major indicator of the microvascular status). In support of these findings similar effects were demonstrated in the experimental animal model with diabetes in the early state (Johnson, 1979A; Gerrard et al, 1980; Rosenblum et al, 1981).

Blood vessels from patients with diabetes and from diabetic rats have been found to synthesise less prostacyclin than vessels from normal subjects (Colwell et al, 1983; Gerrard et al, 1980 and Johnson et al, 1979B) reflecting an altered endothelial function in diabetics. Since it has been suggested that prostacyclin may protect the endothelium against platelet deposition, perhaps an altered balance of prostacyclin/thromboxane in diabetics may in fact promote the onset of vascular disease.

A number of circulatory substances relevant to coagulation including lipids (Stuart et al, 1981), fibrinogen and the von Williebrand factor (Lamberton et al, 1984) have been found to be abnormal in diabetic patients. An increased cholesterol incorporation in the platelet membrane has been reported in diabetics. This may promote increased release of arachidonate from platelet phospholipids and hence increase thromboxane production. If there is a direct relationship between high cholesterol and increased thromboxane, the platelet hypersensitivity could well be a consequence of abnormal plasma lipid concentrations.

Non-enzymatic glycosylation of collagen in diabetics may be another contributory factory in platelet hypersensitivity (Le Pape et al, 1983). Glycosylated collagen from diabetics is a more potent platelet aggregating agent than collagen from control subjects. It is possible that overglycosylated collagen in the vessel walls of diabetics could enhance platelet adhesion, aggregation and the release of platelet granule contents at the site of vessel injury. Although the effect of overglycosylated collagen on platelets may play a part in the development of vascular disease in diabetics, it will not be related to platelet hypersensitivity to other aggregating agents.

Shortened platelet survival and platelet hypersensitivity have been reported in diabetes and other conditions (angina pectoris, hypertension and myocardial infarction) that are associated vessel injury and atherosclerosis (Mustard & Packham, 1977). The precise relationship is not clear. Perhaps an increased rate of platelet clearance could result from increased platelet adherence at the site of vessel injury and lead to increased platelet turnover.

Beta-thromboglobulin (βTG), a specific platelet protein which is stored in platelet α granules is released to the surrounding plasma during platelet aggregation. Its presence in the circulation reflects 'in vivo' platelet activation. Elevated β-thromboglobulin levels have been found in diabetic blood, including newly diagnosed diabetics free from angiopathy (Preston et al, 1978; Betteridge et al, 1981; Burrows et al, 1978).

These findings are indeed in favour of a role of apparent altered platelet/endothelial function in the genesis of vascular complications and added support is gained from reports that platelet microthrombi are evident in retinal microvessels very early on in the diabetic state (Ishibashi et al, 1981).

If there is a direct causal relationship between abnormal platelet/endothelium function and vascular disease, it would be hoped that pharmacological intervention to reduce platelet and endothelial abnormalities, would delay or prevent the occurrence of diabetic vascular disease.

In choosing suitable therapy for the prevention or delaying of vascular disease, the underlying mechanism of enhanced platelet aggregation must be established. The most consistent finding has been the increased production of aggregation-induced thromboxane A_2 (Halushka et al, 1981; Silberbauer et al, 1981). Most studies to date have employed arachidonate or collagen (which releases endogenous arachidonate) to demonstrate enhanced platelet aggregation (Petersen and Gormsen, 1978; Davis et al, 1978). However, it cannot be determined from these studies whether improved thromboxane receptor coupling or an increase in the number of thromboxane receptors may also contribute to this increased sensitivity.

The first study (Study I) in this chapter was therefore aimed at investigating the changes at the receptor level in platelets from diabetics with established micro-angiopathy. Using a stable thromboxane mimetic, 11,9-epoxymethanoPGH$_2$ which directly stimulates

the thromboxane receptor, it was possible to compare the sensitivity of the platelets at the receptor level from diabetic patients to those of control subjects. Platelet sensitivity to collagen was also investigated to confirm platelet hypersensitivity to this agent.

Although the intervention at the platelet/endothelial level is one possible approach to control vascular complications, it is possible that careful metabolic control may restore platelet function to normal in diabetic patients. Beneficial effects of insulin therapy on platelet function include a fall in β-thromboglobulin levels (Preston et al, 1978) and a decrease in whole blood aggreg ability (Juhan et al, 1982). However several observers have reported an increase in sensitivity of platelets to aggregating agents 'in vitro' after insulin (Janka and Demmel, 1981; Hilsted et al, 1980). Due to the inconsistent findings it is therefore still not clear whether metabolic control does restore platelet function to normal. Inconsistencies may arise because of the diverse methods employed in studying platelet function and the difficulty in maintaining a close control over diabetic groups. The aim of the second study (Study II) was therefore to investigate whether there were any changes in certain aspects of platelet function (platelet sensitivity to the aggregating agent, ADP, the anti-aggregatory agent, Iloprost, thromboxane production and adenylate cyclase activity reflected by cyclic AMP levels) in two groups of young insulin-dependent diabetics in which glycaemic control was significantly improved in one group.

RESULTS

Study I - Platelet Thromboxane Receptor Sensitivity in Healthy Volunteers and Diabetic Patients with Background and Proliferative Retinopathy

Patients

The patients studied were type I (insulin-dependent) diabetic patients, 12 with background and 12 with proliferative retinopathy. These patients were compared with 12 non-diabetic healthy volunteers. The three groups were comparable for age and sex, and the diabetic groups were matched for duration of diabetes. Blood glucose levels, percentage haemoglobin A (HbA), and platelet counts were measured at the time of study and no significant difference was found between the two diabetic groups. These parameters are summarised in Table 5.1. Persistent hyperglycaemia modifies (glycosylates) haemoglobin A and these levels reflect blood glucose concentrations. Unlike blood glucose levels,glycosylated haemoglobin concentration (HbA_1) do not fluctuate from hour to hour and give an indication of blood glucose levels up to four weeks before the sample was taken. They are therefore useful in a long-term assessment of control.

Classification of retinopathy was based upon opthalmoscopic and fluorescein angiographic appearances in all patients. In the background retinopathy group, three patients had scattered microaneurysms and haemorrhages, seven patients had hard exudative changes and two patients had ischaemic preproliferative changes.

Table 5.1 Average age, duration of diabetes, plasma glucose, HbA_1 and platelet counts in control and diabetic retinopathy groups of Study I.

Group	Number of Subjects	Average age (yrs)	Duration of diabetes (yrs)	Plasma glucose (mmol/l)	HbA_1(%)	Platelet count ($x10^9/l$)
Control	12 (6F, 6M)	26.7 ± 1.3	-	4.6 ± 0.3	7.6 ± 0.3	274 ± 11
Background Retinopathy	12 (6F, 6M)	27.6 ± 0.9	15.2 ± 1.1 NS	10.5 ± 1.0 NS	11.8 ± 0.7 NS	257 ± 16 NS
Proliferative Retinopathy	12 (6F, 6M)[a]	27.0 ± 1.5[a]	14.6 ± 1.7[b]	11.6 ± 1.7[b]	12.6 ± 0.7[b]	245 ± 12[b]

Results expressed as mean ± s.e.m. [a] NS between the three groups; [b] NS between the diabetic groups

NS - no significance between diabetic and control groups.

None of the diabetic subjects were taking any medication other than insulin, all were clinically free of nephropathy (urine Albustix negative and plasma creatinine less than 150μmol/l) and all had easily palpable peripheral pulses. Blood was collected between 0800 and 0900 hours from fasted diabetics and control subjects.

Results I

A comparison of dose-response curves for aggregation induced by 11,9emPGH$_2$ in the presence and absence of froben (10μM) and EP092 (0.25μM) in control and platelets from diabetic donors was carried out. The nature of the aggregatory waves in control and diabetic subjects was similar.

11,9emPGH$_2$ acts directly at the platelet thromboxane receptors to induce platelet aggregation. Secondary aggregation partly results from the stimulation of granule release by 11,9-emPGH$_2$ itself and partly from a small contribution by an endogenous thromboxane component. In the presence of Froben the latter component is eliminated (Fig.5.1). In the presence of EP092, the dose-response curve for aggregation induced by 11,9-emPGH$_2$ was shifted to the right giving an indication of the affinity of EP092 for the receptor.

Similar dose response curves were established using collagen as the agonist. Shifts in the dose response curves were established in the presence of EP092 and Froben. In these studies collagen was left in the platelet suspension for 4 minutes, since the onset of aggregation is characteristically delayed. Unlike the effect on

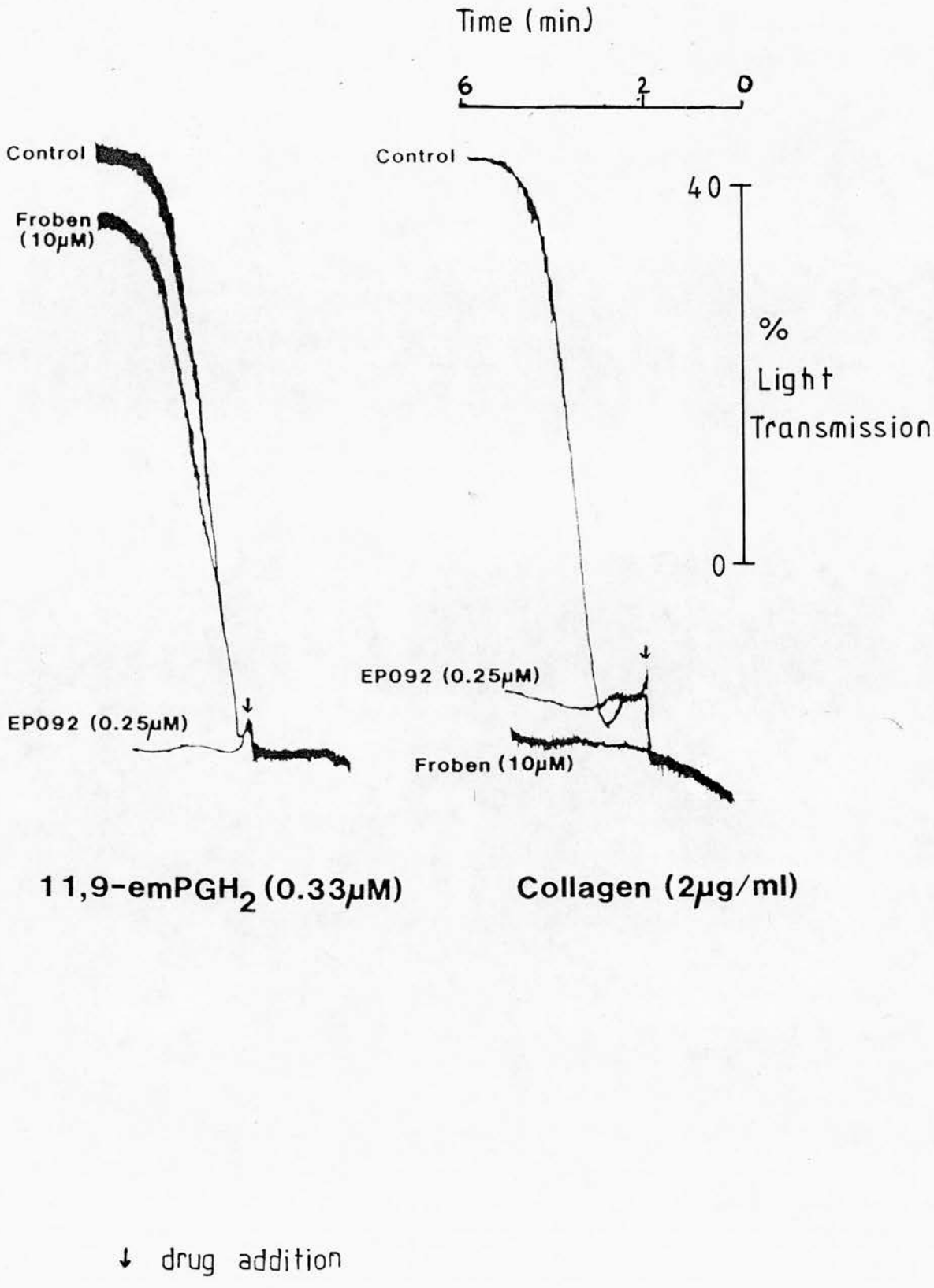


Figure 5.1 Typical aggregation curves observed in human PRP (control donors). Aggregation is induced by 11,9-epoxymethanoPGH$_2$ and collagen in the presence of EP092 (0.25μM) and Froben (10μM). Collagen was incubated for 4 minutes.

11,9-emPGH$_2$ induced aggregation, Froben blocks AA metabolism and totally abolished aggregation induced by the supramaximal dose of collagen (2μg/ml). EP092, the TX receptor blocker, also produces complete inhibition of the response indicating a marked effect of the endogenous TX-endoperoxide component (Fig.5.1).

From the log dose response curves for collagen and 11,9-emPGH$_2$, the concentration of each agonist required to produce 50% of the maximal response was determined for the control and diabetic groups. There was no significant difference in the concentration of collagen required to give 50% of the maximum aggregation wave (EC_{50}) between the two diabetic groups and the control group. In the presence of the thromboxane receptor antagonist EP092 (0.25μM), log dose response curves for collagen were shifted to the same extent in the three groups, as shown by the new EC_{50} values. No significant differences in the dose ratios were evident. The cyclo-oxygenase inhibitor Froben (10μM) produced a greater shift EP092 of the control aggregation curves than EP092. However, there was no significant difference in the shifts with EP092 in the 3 groups and although Froben appeared to be slightly less effective in the proliferative retinopathy group, the difference did not reach statistical significance (Table 5.2).

There was a significant difference between the 3 groups in the concentration of 11,9-emPGH$_2$ required to induce a 50% aggregation response. The proliferative retinopathy group were most sensitive to 11,9-emPGH$_2$ and as a result the EC_{50} value was less than in either the control or background retinopathy groups ($p < 0.005$).

Table 5.2 Results of aggregation EC_{50} and dose ratio for collagen and 11,9-emPGH$_2$ alone and in the presence of EP092 (0.25μmol/l) and Froben (10μmol/l).

Group	Collagen EC_{50} (μg/ml)	Collagen + EP092 EC_{50} (μg/ml)	Dose Ratio	Collagen + Froben EC_{50} (μg/ml)	Dose Ratio	11,9emPGH$_2$ EC_{50} (ng/ml)	11,9emPGH$_2$ + EP092 EC_{50} (ng/ml)	Dose Ratio	11,9emPGH$_2$ + Froben EC_{50} (ng/ml)	Dose Ratio
Control	0.8±0.1	1.9±0.3	2.4±0.2	8.3±1.9	9.8±2.0	99.4±10.0	115.3±5.2	1.7±0.1	124.7±9.4	1.31±0.1
Background Retinopathy	0.74±0.1	1.8±0.3	2.7±0.5	6.6±1.6	10.8±3.4	85.4±11.5	193.8±28.4	2.6±0.6	120± 2.9	1.51±0.4
Proliferative Retinopathy	1.1±0.2	1.9±0.4	1.9±0.2	4.9±0.7	5.0±0.5	63.5±6.6	176.0±20.2	3.15±0.6	100.4±8.8	1.62±0.1
Significance	NS	NS	NS	NS	NS	p<0.005	NS	p<0.005	NS	NS

(Results expressed as mean ± s.e.m.)

p<0.005, significant difference between proliferative retinopathy group and background retinopathy group

Furthermore EP092 produced a significantly larger shift in the aggregation curve in the proliferative retinopathy group as judged by the dose ratio when compared with the other 2 groups ($p < 0.005$). It may be of interest that in the presence of Froben, no difference was found between the 3 groups in either the EC_{50} or dose ratio.

Release of 5HT was simultaneously measured during these aggregation experiments (Table 5.3). No difference was observed in the release of 5HT in response to both collagen and 11,9emPGH$_2$ between the three groups. Basal cyclic AMP levels and the elevation in cyclic AMP induced by PGE_1 (0.2μM) were also measured. No significant difference was observed in the basal levels or the rise in the 3 groups (Fig.5.2).

Statistical analysis of the dose ratios was by Kruskal-Wallis one-way analysis of variance by ranks. Comparison of cyclic AMP levels between the groups was carried out using the independent Student's t-test.

Study II - Improvement of Glycaemic Control

Patients

Nine moderately-well controlled male insulin-dependent diabetics underwent platelet function tests at time 0 (To) and again after six months (T_1). Five of these patients (tight control group) improved their glycaemic control as indicated by changes in their glycosylated haemoglobin over the six month period, while four ("usual" control

Table 5.3 Results of platelet release action in control and diabetic retinopathy groups due to collagen (2µg/ml) and (250ng/ml) 11,9-emPGH$_2$ alone and in the presence of EP092 (0.25µmol/l) and Froben (10µmol/l)

Group	Collagen	Collagen + EP092	Collagen + Froben	11,9-emPGH$_2$	11,9-emPGH$_2$ + EP092	11,9-emPGH$_2$ + Froben
Control	58.8 ± 2.2	41.1 ± 3.6	19.8 ± 1.1	34.9 ± 1.0	30.9 ± 1.4	32.7 ± 1.3
Background Retinopathy	51.8 ± 4.5	39.3 ± 3.9	21.6 ± 1.4	30.8 ± 2.9	26.7 ± 2.9	29.3 ± 2.3
Proliferative Retinopathy	55.3 ± 5.7	30.7 ± 4.1	18.4 ± 2.4	32.8 ± 2.4	28.7 ± 3.4	30.1 ± 1.7

Results expressed as % total [^{14}C]-5-HT released/[^{14}C]-5-HT taken up by platelets at pre-incubation and given as mean ± s.e.m.

group) were unable to improve their glycaemic control (Table 5.4). Opthalmoscopy following mydriasis showed three patients in the 'tight control' group and two patients in the 'usual control' groups to have mild background retinopathy. All were clinically free of nephropathy (urine Albustix negative and plasma creatinine less than 150mol and all had easily palpable peripheral pulses. None were taking medication except insulin and none had experienced a hypoglycaemic reaction within 24 hours of venesection. Blood was withdrawn from fasting diabetic subjects between 0800 and 0900 hours.

Results II

Log dose response curves were established for ADP induced aggregation using platelets from nine subjects before and 6 months after a period of glycaemic control. The concentration of ADP required to produce 50% of the maximum aggregation wave response (EC_{50}) was esentially unchanged following 6 months of glycaemic control (Table 5.5). In the presence of the prostacyclin mimetic, Iloprost (1nM) the log dose response curves for ADP were shifted to the right. Before improvement of glycaemic control (T_0) the shift of the curves to the right was similar in both groups. However, after 6 months (T_1) there was a significant increase in the responsiveness of the platelets to Iloprost in the 'tight' control group, reflected by a much greater shift to the right of the log dose response curve for ADP (Table 5.5).

Thromboxane B_2 production in response to exogenous collagen (2µg/ml) was measured. There was no significant difference in TXB_2

Table 5.4 Clinical details of patients in study II

Group	Age (yrs)	Duration (yrs) of diabetes	HbA_1 (%) (T_0)	HbA_1 (%) (T_1)
"Tight" control	30.8	8.8	11.9	9.0
(n = 5)	(28.6-32.8)	(6.2-10.6)	(10.1-12.8)	(7.6-10.1)
"Usual" control	29.8	8.6	11.4	11.9
(n = 4)	(29.9-32.1)	(6.3-9.5)	(9.6-12.2)	(9.4-13.0)

Results are expressed as mean (range).
(HbA_1, normal range 6-8%).

Table 5.5 Platelet aggregation EC_{50} for ADP, dose ratio for inhibition by Iloprost (1.5×10^{-9}M) and platelet cAMP level (pmol cAMP/ml PRP) following exposure to Iloprost (1.5×10^{-9}M) after improved glycaemic control in study II

Groups	EC_{50} (10^{-7}M)		Dose-ratio for inhibition by Iloprost		Platelet cAMP level after exposure	
	T_0	T_1	T_0	T_1	T_0	T_1
"Tight" control	8.0±2.3	6.0	1.25	3.3*	1.23	1.21
(n = 5)	(5.4±10.2)	(3.2-8,3)			(1.02-1.38)	(0.99-1.62)
"Usual" control	6.0	6.5	2.50	1.9	1.31	1.2
(n = 4)	(4.1-8.6)	(4.2-8.8)			(1.04-1.68)	(1.0-1.45)

Results expressed as mean (range). (*p < 0.05)

levels before glycaemic control in the two groups. However, a significant increase ($p < 0.05$) was apparent at T_1, in the group whose glycaemic control had improved (Fig.5.3).

Cyclic AMP levels both basal and after exposure to Iloprost were measured in the two groups at T_0 and T_1 (Table 5.5). No significant difference was apparent in these values.

Statistical analysis was performed using the Wilcoxon rank sum signed test.

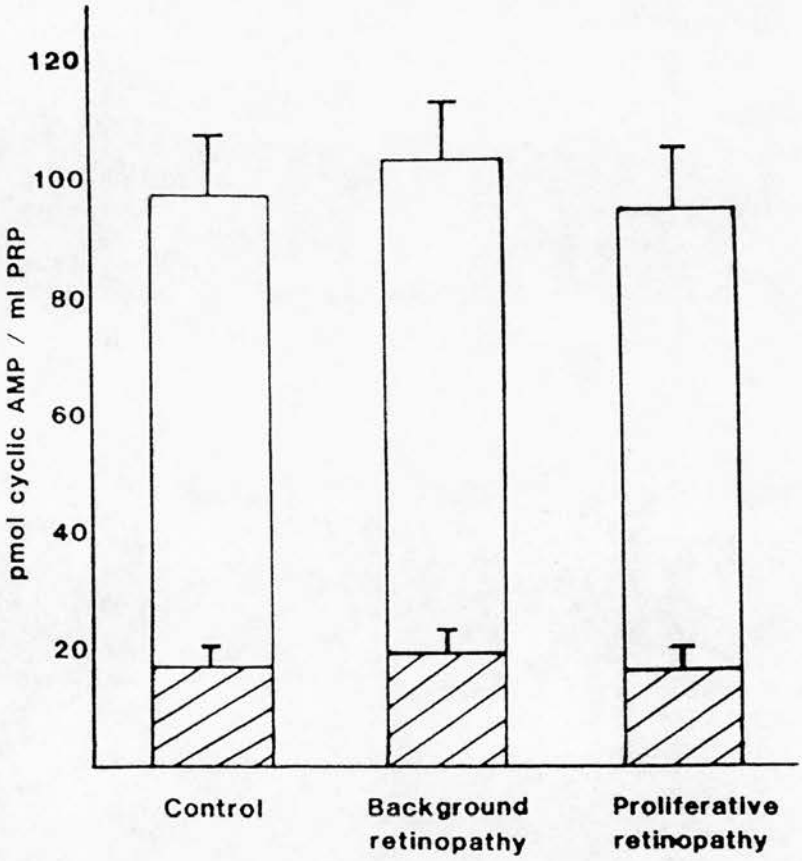


Figure 5.2 Basal platelet cyclic AMP levels and cyclic AMP levels after exposure to prostaglandin E_1 in control in diabetic retinopathy groups. Results are given as the mean ± s.e.m. n = 12 in all groups.

▨ = basal cyclic AMP level

□ = cyclic AMP level after exposure to PGE_1.

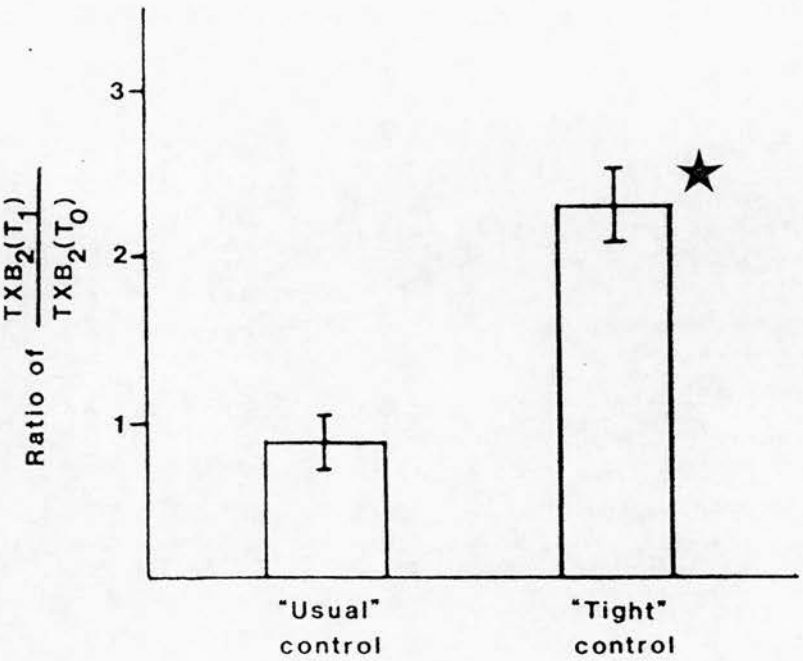


Figure 5.3 Ratio of platelet TXB_2 production at 6 months (T_1) with platelet TXB_2 production at start of the study (T_0) in the "usual control" and "tight control" groups.
* $p < 0.05$ (mean ± s.e.m.).

DISCUSSION

The results from study 1 demonstrate that platelets from Type I diabetics with proliferative retinopathy are slightly more sensitive to the thromboxane mimetic 11,9-emPGH$_2$ than platelets from Type I diabetic patients with background retinopathy and control subjects. In the presence of Froben the increased sensitivity to 11,9-emPGH$_2$ was reduced. In non-diabetics subjects the major mechanism of the action of the mimetic does not involve activation of the thromboxane generating system (Hamberg et al, 1975; Moncada and Vane, 1978). It would appear therefore that since Froben eliminates the endogenous thromboxane component and reduces the sensitivity to 11,9-emPGH$_2$, then the increased sensitivity in the proliferative group is not a direct effect of the 11,9-emPGH$_2$ at the receptor. The demonstration that in the presence of the thromboxane receptor blocker, EP092 the shift of the dose response curves to the right were similar in the three groups, indicates that the affinity of EP092 for the receptor is similar in the 3 groups.

The mechanism of increased sensitivity in the proliferative retinopathy group remains undefined; the possibility that enhanced release of dense granule constituents namely 5HT, may be responsible for increased sensitivity due to the direct action of 11,9-emPGH$_2$ endogenous thromboxane on the granules can be ruled out, since it is apparent from Table 5.3 that there is no significant different in the release of 5HT induced by either collagen or 11,9-emPGH$_2$ in each of the 3 groups. The increased sensitivity, therefore may be due to an increased endogenous thromboxane production ocurring during secondary

aggregation.

In contrast to previous studies with diabetic patients (Silberbauer et al, 1981; Sagel et al, 1975; Jones et al, 1985A and Janka and Demmel, 1981) enhanced platelet sensitivity to collagen was not apparent in this study whether measured as platelet aggregation or as 5HT release. In non-diabetic subjects endogenous PGH_2 and thromboxane A_2 production are the major mediators of collagen-induced aggregation (Vargaftig et al, 1981). The aggregation EC_{50} values for collagen were similar in the 3 groups. The thromboxane receptor antagonist EP092 produced a similar shift of the aggregation curve in each group. The cyclo-oxygenase inhibitor, Froben appeared to be slightly less effective in inhibiting collagen induced aggregation in the proliferative group indicated by smaller shift of the dose response curves. Although this effect did not reach statistical significance, the decreased activity of Froben in this group may be expected if there was an increased endogenous thromboxane synthesis, although in this instance it is not apparent in the aggregation EC_{50} values. It is clear from Table 5.3 that collagen is a more effective stimulator of the release reaction and since a marked reduction in release is evident in the presence of Froben, endogenous TXA_2 and the endoperoxides must play a major part in collagen induced release of 5HT. Since no statistically significant differences were demonstrated either in EC_{50} values or 5HT release values in response to collagen, it would seem likely that the mechanism underlying collagen-induced aggregation is similar in platelets from diabetics to those of control groups.

The apparent similarity in platelet sensitivity to collagen between the 3 groups in this study contrasts with other studies (Silberbauer et al, 1981; Sagel et al, 1975; Jones et al, 1985A; Janka and Demmel, 1981). These discrepancies may be accounted for by the careful age restriction and comparability of subjects during recruitment to the study. Age is an important determinant of platelet function in both non-diabetic and diabetic patients (Leccrubier et al, 1980).

In addition no difference was observed between either the basal level of platelet cyclic AMP or the elevated level of cylcic AMP following exposure to prostaglandin E_1 in both the diabetic and control groups. This finding is in agreement with a recent study of prostacyclin binding to platelets and activation of adenylate cyclase (Shepherd et al, 1983) which showed similar PGI_2 binding and adenylate cyclase activity of human platelets from proliferative diabetics and control subjects. These reports are in sharp contrast to a report by Lagarde and co-workers (1981A) who found that diabetics had lower resting levels of cyclic AMP and a diminished response to inhibitory prostaglandins when compared to controls. One possible explanation for these discrepancies is that in this study and the one by Shepherd (1983) the subjects were young and age matched (25-28 years and 31-35 years respectively), whereas in the report by Lagarde et al (1981), the duration of diabetes vascular, complications and age range (17-61 years) were very wide.

In summary, this study shows that platelets from young Type I diabetic patients with microvascular disease in the form of

background and proliferative retinopathy are similar to platelets from normal subjects in their response to collagen-induced aggregation. However, diabetics with proliferative retinopathy were found to have platelets with a slightly enhanced sensitivity to the thromboxane mimetic 11,9-emPGH$_2$. Since the increased sensitivity does not appear to be mediated by a direct effect at the receptor level, it is unlikely that thromboxane receptor antagonists would prove to be useful therapeutic drugs to reduce an enhanced sensitivity of platelets to thromboxane in diabetics with proliferative retinopathy. Owing to the absence of differences in platelet sensitivity to collagen in the 3 groups studied, and the slight increase in platelet sensitivity to 11,9-emPGH$_2$ in the proliferative group, it is unlikely that an alteration in platelet function is a major causative factor in the genesis of retinopathy, and may in fact be secondary to the microvascular complication.

The second study deals with two groups of diabetic patients; those that did not improve their glycaemic control over six months ('usual' control group) and those that did improve their glycaemic control ('tight' control group). Although significant improvement of the glycosylated haemoglobin (HbA_1) was obtained, HbA_1 levels in the 'tight' control group did not reach normal levels.

The sensitivity of platelets from nine diabetic subjects was assessed by measuring the aggregation response to ADP and the effect of the anti-aggregating agent, Iloprost. There was a trend of increasing sensitivity to ADP in the group whose glycaemic control was improved, but this change did not achieve statistical

significance. Since platelet sensitivity of the individual fluctuates from day to day, perhaps if the numbers in each group were larger, a significant difference might have been demonstrated. It has been reported (Janka and Demmel, 1981) that during poor metabolic control platelet functions are depressed as reflected by decreased platelet aggregation responses and it has been suggested that during prolonged hyperglycaemia, depletion of platelet glycogen stores and decreased ATP generation could account for depressed platelet function. In addition erythrocytes and leukocytes have been found to exhibit subnormal functions in a state of poor metabolic control (Peterson et al, 1977).

Platelets from the 'tight' control group were found to be significantly more sensitive to the inhibitory agent, Iloprost after improvement of glycaemic control. The dose response curves for diabetics in the tight control group were shifted much further to the right in the presence of Iloprost. At first instance, this would suggest that the increased sensitivity is mediated through raised cyclic AMP levels possibly due to enhanced adenylate cyclase activity or improved receptor coupling. However, no significant difference was found in the cyclic AMP levels measured in plasma after exposure to Iloprost or in the basal levels with the improvement of glycaemic control. It is possible that the increase in Iloprost sensitivity in the 'tight' control group, reflected by a more marked inhibition of aggregation occurs at some site other than the prostacyclin receptor adenyl-cyclase complex; perhaps a more efficient sequestration of cytosolic free calcium.

Platelets from diabetics have been demonstrated to have decreased sensitivity to inhibiting prostaglandins (Betteridge et al, 1982; Davi et al, 1982). Furthermore, 'in vivo' reduced vascular PGI_2 levels have been found at particularly high blood glucose levels (Johnson et al, 1979B, Silberbauer et al, 1979; Aanderud et al, 1985; Wilson and Tan, 1985). It may not be surprising that in this study, subsequent lowering of blood glucose results in an increased sensitivity to inhibiting prostaglandins. Furthermore, Janka and Demmel (1981) also found an increase in adenylate cyclase activity with improvement of glycaemic control in a heterogeneous group of non-insulin and insulin dependent diabetics.

In addition, platelets from diabetics in the tight control group were found to produce more TXB_2 in response to exogenous collagen, following improvement of glycaemic control. Reduced prostaglandin production from endogenous platelet arachidonic acid (Janka and Demmel, 1981) and reduced serum thromboxane levels in diabetics (Tindall et al, 1981; Ylikorkala et al, 1981) have been reported. Jones et al, (1983) have demonstrated reduced arachidonate acid levels in the platelet membrane phospholipids of diabetics which correlated negatively with glycosylated haemoglobin. Thus lowering of glycosylated haemoglobin with improvement of glycaemic control may in fact increase the availability of arachidonic acid from membrane phospholipids (Jackson et al, 1984) and may explain the increased TXB_2 production observed in this study.

The overall picture from this study, shows an increase in sensitivity to Iloprost and an increased production of thromboxane

B_2. Normally enhanced aggregation responses may be expected with an increase in thromboxane production. In this instance perhaps the action of increased thromboxane is opposed by the increased sensitivity of the inhibitory system and this may offer an alternative explanation for the lack of a significant change in the ADP aggregation response accompanying improved glycaemic control. It has been reported that platelets from mature rats are more sensitive to prostacyclin as well as showing increased sensitivity to collagen when compared to young rats (Giani et al, 1985). It is possible that a similar situation operates in diabetics undergoing improvement of glycaemic control whereby an enhanced inhibitory system exists to counteract the effects of increased thromboxane in order to maintain a haemostatic balance.

Results in this study, indicate that short term glycaemic control in a heterogeneous group of insulin dependent diabetics does not markedly change all aspects of platelet function in favour of reducing the prothrombotic state, i.e. increased platelet thromboxane production. It may also be of interest that in the first year at least, improved glycaemic control does not necessarily slow the evolution of mild microangiopathic complications in the form of non-proliferative diabetic retinopathy (Lauritzen et al, 1983; Kroc Study Group, 1983). Indeed in some instances a significant deterioration in retinopathy has been reported (Dahl-Jorgensen et al, 1985; Tamborlane et al, 1982). The present study demonstrates that with improved glycaemic control over six months towards, but short of physiological there is an increased production in the proaggregatory agent, thromboxane A_2 which may be a contributory factor in the

deterioration of microvascular disease, particularly in the form of retinopathy. However, the increased sensitivity to prostacyclin may in fact reflect a compensatory mechanism in the body to counteract the increased proaggregatory factors.

At present, it would be wise not to expect short-term control to eliminate the risk of thrombosis. However, it remains to be investigated whether treatment achieving long term control may prove to be the desirable way to lessen the risk of developing further vascular disease and related thrombotic complications. One must bear in mind that in addition to the evidence which suggests that platelet/endothelial functions contribute to thrombo-embolic complications, perhaps the mode of therapy (i.e. improving glycaemic control) employed to reduce the hyperglycaemic state may also be a causative factor in these conditions.

In this clinical study the control subjects were healthy volunteers working within our university department. It should be emphasised however that control subjects are not a random representation of the population. The use of such control groups from a narrow cross-section of the population may be of importance in this study since there are likely to be marked differences in the dietary and social habits of these individuals to those in diabetic groups. The individuals diet, alcohol consumption, smoking habits, etc. were not considered when choosing controls in this study. Since all of these factors as well as others can affect platelet function there are limitations in comparing these control groups with diabetics. If a random cross-section of subjects were chosen as controls perhaps a truer representation of the population would be achieved. Furthermore if daily fluctuations in individuals' platelet function were measured, perhaps one could assess the validity of comparing control groups to diabetics over a period of time.

During the studies in this thesis because time was a limiting factor it was not possible to choose a control group representing a true cross-section of the population.

Chapter 6

Hypothyroidism and platelet function

INTRODUCTION

Hypothyroidism and thyrotoxicosis, like diabetes mellitus are associated with coronary heart disease and other complications. Hypothyroid patients have been shown to have twice the incidence of severe coronary atherosclerosis compared to controls matched for age, sex and blood pressure (Vanhaelst et al, 1967). There has been much discussion as to whether hypercholesterolemia that accompanies primary hypothyroidism accelerates the development of coronary atherosclerosis. Necropsy data suggests that hypercholesterolemia in hypothyroid patients predisposes coronary atherosclerosis but only in the presence of hypertension. In normotensive patients the degree of atherosclerosis is no greater than in age-and sex-matched normotensive controls (Steinberg, 1968). Despite hypercholesterolemia and increased atherosclerosis the incidence of myocardial infarction in untreated hypothyroids is not increased. However, it has been demonstrated that when patients are given thyroxine, the incidence of angina pectoris and the risk of myocardial infarction increases (Wayne, 1960). In addition to these problems, haematological abnormalities have also been observed in association with hypothyroidism, including easy bruising, menorrhagia and bleeding tendancies. It is possible that thyroid function disorders, in particular hypothyroidism, may be associated with a haemostatic imbalance. In response to diminished oxygen requirements and decreased erythropoietin, red cell mass is decreased. Furthermore, the intrinsic clotting mechanism has been found to be defective in the hypothyroid state (Egeberg, 1963, 1964) and a decrease in the plasma concentration of factors VIII and IX

together with an increase in capillary fragility may be responsible for the bleeding tendancies reported in hypothyroids (Simone et al, 1965). Furthermore changes in both plasma constituents and erythrocyte deformability have been found which may be responsible for increasing blood viscosity (Larsson et al, 1985) and altering red cell shape (Wardrop and Hutchison, 1970).

Several groups have shown fibrinolytic activity to be increased when thyroxin levels are below normal (Bennet et al, 1967; Hume, 1965; Jacobsen, 1968) and it has been suggested that a depression of the fibrinolytic activity following administration of thyroxin may facilitate the formation of an occluding thrombus in coronary arteries.

The close association of haematological disorders and hypothyroidism may indicate a possible role for platelets in these abnormalities. Since platelet counts are normal in hypothyroidism (Egeberg, 1963), platelet disfunction is likely to be of a qualitative nature. In fact, it has been demonstrated that platelet adhesivensss is reduced in hypothyroidism and only increases to normal after thyroid hormone replacement therapy (Hellem et al, 1975). Although, clear haematological disorders, including some aspects of platelet function are associated with hypothyroidism, very few platelet studies have been carried out on hypothyroid patients. One study has shown that platelets from hypothyroids responded very weakly to various aggregating agents including ADP, adrenaline and collagen, measured by the turbidometric aggregation method of Born (1962). Replacement therapy with thyroxine (T_3) rendered these

platelets hyper-responsive to the aggregating agents above (Gardikas et al, 1972).

Contrasting effects are observed in thyrotoxic patients whose thyroid glands have become overactive. In such conditions, the plasma cholesterol levels are low, the intrinsic coagulation systems overactive and the fibrinolytic system are depressed. In addition, platelet abnormalities have been demonstrated; including increased platelet adhesiveness (Hellem et al, 1975), a decreased platelet count and a reduced platelet life span (Lamberg et al, 1971). In view of these abnormalities, an enhanced risk of platelet thrombus formation may be expected. However, the incidence of atherosclerosis in hyperthyroid patients is very low and this has been attributed to the anti-atherogenic effct of the thyroid hormone (Myasnikov et al, 1963). This group demonstrated that in cholesterol-fed rabbits treatment with thyroid hormones lowered serum cholesterol levels and increased the coronary blood flow consequently reducing local thrombus formation. Thus the anti-atherogenic effect of the thyroid hormone and low cholesterol levels associated with thyrotoxicosis are in favour of preventing thrombotic tendancies.

Thyrotoxicosis may be treated in a number of ways; by surgical removal of part of the gland, carbimazole treatment (which involves inactivation of thyroid stimulating hormone) and the use of radio-active iodine. The use of radio-active iodine is the most common method employed especially in the early stages of thyrotoxicosis. However, it is often difficult to predict the dose of radioactive iodine to administer. In these circumstances the emergence of

clinical hypothyroidism often results and thyroxin replacement therapy is necessary to increase thyroid levels to the euthyroid state. When undergoing replacement therapy the patient is at risk of developing angina pectoris or a myocardial infarction (Wayne, 1960). In fact, the institution of thyroxine replacement therapy once clinical hypothyroidism has emerged has been suggested to be associated with significant morbidity, especially in older patients who constitute a large percentage of the hypothyroid population (Rosenbaum and Barzel, 1981; Bahemuka and Hodkinson, 1975). Enhanced platelet sensitivity accompanying treatment could also contribute to the risk of coronary arteriosclerosis, angina pectoris and myocardial infarction.

This study was designed to investigate platelet function in a group of clinically hypothyroid patients before and after replacement therapy. Platelet sensitivity was measured using two differently acting aggregating agents, ADP and the thromboxane mimetic, 11,9-epoxymethanoPGH$_2$. It is thought that platelet reactivity is controlled to some extent by a thromboxane-prostacyclin balance and an imbalance of these two arachidonate metabolites may lead to abnormal platelet function. For these reasons, both TXB$_2$ production and cyclic AMP levels (which reflect prostacyclin activity) were determined before and after treatment with the thyroid hormone.

RESULTS

Blood (100ml) was collected from patients on the morning of the experiment and platelets (as PRP) were prepared as described in the methods chapter. Five patients were studied, two of whom had Grave's disease and three who were diagnosed with multinodular goitre. All the patients were thyrotoxic and became hypothyroid after radioactive iodine. The clinical details of each of the patients are shown in Table 6.1.

Platelet aggregation studies were carried out before (0 mths) and after (6 mths) thyroxine replacement therapy. Log concentration response curves were established for ADP and the thromboxane mimetic, 11,9-epoxymethanoPGH_2. From these curves the concentration of each agonist to produce a 50% maximal response (EC_{50} value) were determined (Fig.6.1).

It is apparent from Fig.6.1A that there is an increase in platelet sensitivity to ADP after treatment at the 6 month period reflected by a decrease in the EC_{50} values. This increased platelet sensitivity is statistically significant ($0.05 > p > 0.02$). Although there is also a reduction in the EC_{50} values for 11,9-emPGH_2 induced aggregation at 6 months, the difference did not reach statistical significance.

Thromboxane B_2 production in response to exogenous collagen (2µg/ml) was measured before and after treatment. PRP was incubated with collagen for 60 seconds at 37°C. The reaction was terminated

Table 6.1 Clinical data, thyroid function tests, cholesterol levels and platelet count before (0 mths) and after (6 mths) replacement therapy with thyroxine (dosage indicated).

		Before (0 mths)					After (6 mths)					
Patient	Age (yrs)	T_3 (nmol l^{-1})	T_4	TSH mUl^{-1}	Cholesterol mmol l^{-1}	Plat.Count $x10^9l^{-1}$	T_3 (nmol l^{-1})	T_4	TSH mUl^{-1}	Cholesterol mmol l^{-1}	Plat.Count $x10^9l^{-1}$	Thyroxine Dosage(µg)
1	46	0.8	28	62	7.0	290	1.7	138	4.8	4.9	243	150
2	60	1.1	<20	57	11.9	316	1.9	151	<1.0	7.2	432	200
3	34	0.8	18	103	10.4	433	1.8	149	5	5.3	355	150
4	56	0.6	<20	206	4.2	338	1.7	136	4.1	2.0	291	200
5	45	0.5	<23	77	9.8	305	1.8	140	2.3	6.2	318	150

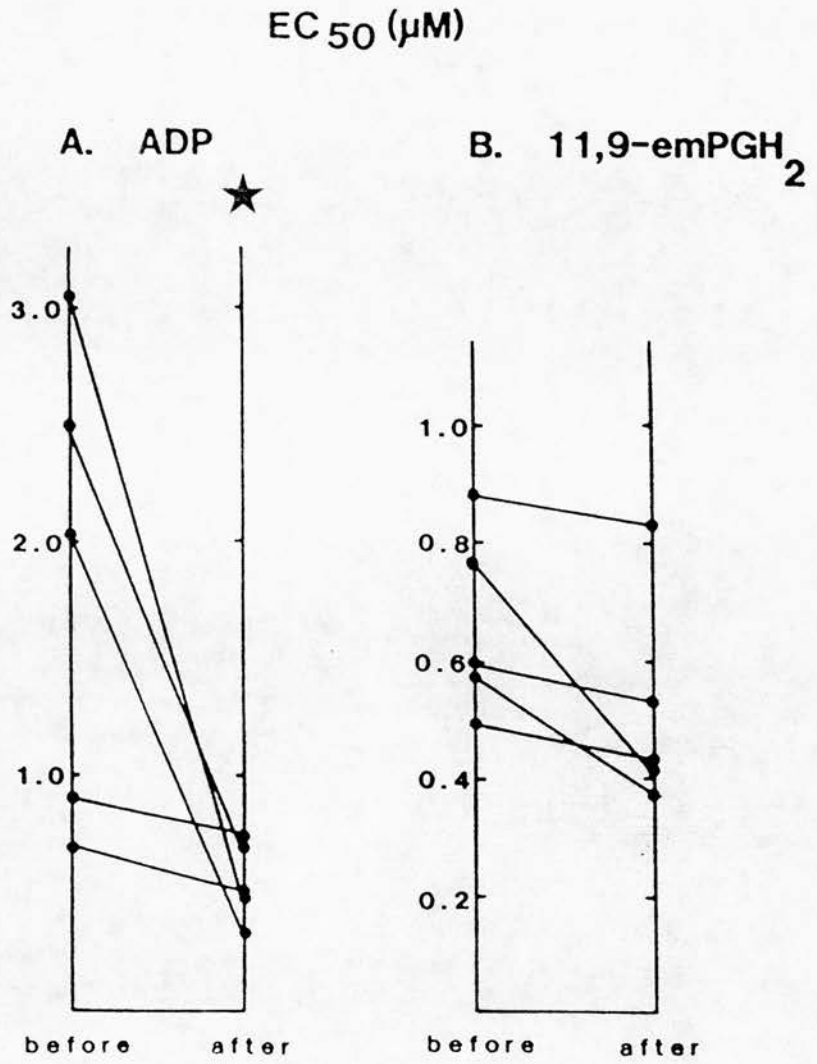


Figure 6.1 The EC_{50} values (μM) for platelet aggregation induced by (A) ADP and (B) 11,9-epoxymethanoPGH$_2$ before and after thyroxine treatment. * $0.05 > p > 0.02$.

and TXB_2 was extracted as described in the methods chapter. There was no statisticlly significant difference in the thromboxane levels before and after 6 months of thyroxine treatment (Fig.6.3).

Elevations in cyclic AMP levels in response to Iloprost (0.03μM) were measured at 0 months and 6 months (Fig.6.2). There is a marked fall in elevated cyclic AMP levels after thyroxine treatment which reached statistical significance ($0.05 > p > 0.02$). There was no significant difference in basal levels of cyclic AMP before (12.25 ± 1.02) and after (16.00 ± 2.92) thyroxine treatment. Statistical tests were carried out using the Wilcoxon rank sum test.

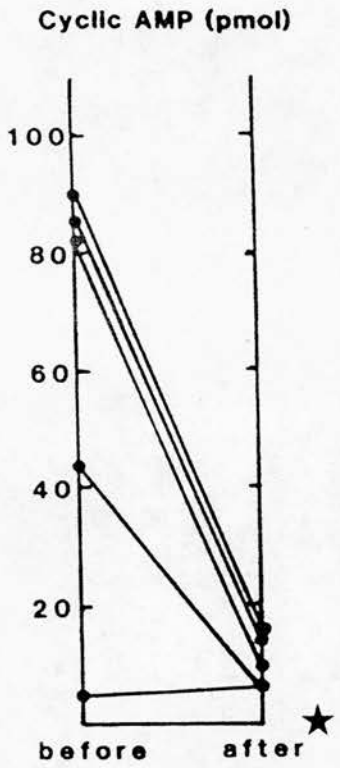


Figure 6.2 Cyclic AMP production (pmol/ml of PRP) after exposure to Iloprost (0.03μM) before and after thyroxine treatment.
* $0.05 > p > 0.02$

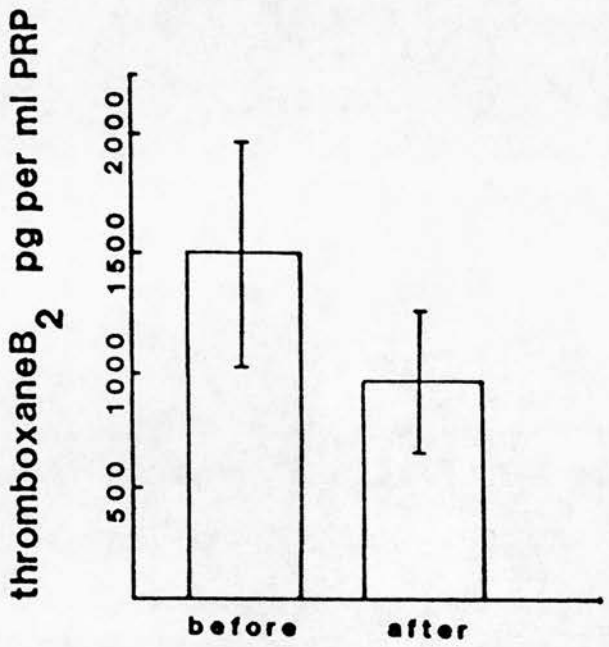


Figure 6.3 TXB_2 (pg/ml of PRP) production in response to collagen (2µg/ml) (incubated for 60 seconds) before and after treatment with thyroxine.
Values are the mean ± s.e.m. from 5 patients.

DISCUSSION

It appears from this study that thyroxine treatment in hypothyroidism is associated with enhanced platelet aggregation. The increased sensitivity after six months of replacement therapy is more marked with ADP than 11,9-epoxymethanoPGH$_2$ and only the former aggregating agent induces a change which is statistically significant.

There is a much greater scatter in the ADP EC_{50} values (0.7 - 3.0μM) before treatment when compared with those of 11,9-epoxymethanoPGH$_2$ (0.5 - 0.9μM). The number of patients studied was small and individual or daily fluctuations may contribute to the scatter observed in ADP EC_{50} values. However, the scatter was markedly reduced following treatment. The two extreme ADP EC_{50} values before treatment correlate with the 2 extreme values of T_3 levels before treatment. It is possible that platelet sensitivity to ADP is more susceptible than 11,9-emPGH$_2$ to metabolic changes (such as T_3 levels) and the scatter of ADP EC_{50} values may be related to T_3 levels and the degree of hypothyroidism in the patient. There does not appear to be any correlation between the ADP EC_{50} values and those of 11,9-emPGH$_2$ (i.e. the individuals most and least sensitive to ADP do not correspond to those most and least sensitive to 11,9-emPGH$_2$). This may suggest that the metabolic changes associated with hypothyroidism affect platelet sensitivity to ADP and 11,9-emPGH$_2$ in different ways.

Indeed the mechanisms underlying the aggregatory response

induced by these agents differ; ADP has a large direct component at an ADP receptor and a contribution from the release reaction. 11,9-EpoxymethanoPGH$_2$ acts directly at an endoperoxide/thromboxane receptor as well as inducing the release of granule constituents either directly or indirectly through endogenous TXA$_2$.

Since the EC$_{50}$ values presumably reflect mainly primary wave aggregation, the increased ADP sensitivity may be a consequence of improved stimulus-response coupling at the platelet ADP receptor. The finding that thromboxane B$_2$ production in response to collagen was not significantly altered after thyroxine treatment, may indicate that there is not an enhanced metabolic conversion of arachidonate or increased arachidonate availability. Whether there is a more efficient triggering of the release reaction would not be apparent from comparing EC$_{50}$ values but it is possibile that second phase aggregation may also be enhanced due to an improved release reaction. Measurement of products of 'in vitro' release would show whether this was the case or not.

Although a significant decrease in the elevated cyclic AMP levels following thyroxine treatment is evident, this fall is unlikely to be a factor contributing to the decreased ADP EC$_{50}$ values in the 'in vitro' system. Since ADP has a tendancy to inhibit the adenylate cyclase enzyme (Haslam et al, 1979) and prostacyclin enhances it, a reduction in the activity of this enzyme system may cause the basal levels of cyclic nucleotides to be depressed, which could account for enhanced aggregatability 'in vitro'. In this instance, the basal levels of cyclic AMP before and after treatment

are not significantly different. It would seem therefore that 'in vitro' cyclic AMP levels are not contributing to the enhanced 'in vitro' platelet sensitivity to ADP.

Although the marked reduction in the prostacyclin-stimulated cyclic AMP levels would not be of any consequence in the 'in vitro' platelet system a similar reduction 'in vivo' may have considerable implications. The thromboxane/prostacyclin balance is one of a number of factors governing platelet reactivity (Gorman et al, 1977). If platelet reactivity 'in vivo' is controlled by a prostacyclin feedback mechanism, depressed activity of the prostacyclin receptor/adenylate cyclase complex with subsequent reductions in cyclic AMP levels would perhaps be reflected by enhanced thromboxane activity.

The enhanced sensitivity to ADP, observed 'in vitro' following thyroxine treatment may be of relevance 'in vivo'. It has been reported that thyroxine promotes various metabolic actions of adrenaline (Gardikas et al, 1972). In vivo a synergy between the increased sensitivity to ADP and enhanced activity of adrenaline may contribute to hypersensitivity of platelets. These factors as well as a supressed receptor-adenylate cyclase complex may be major contributory parameters in the development of vascular complications following thyroxine treatment.

In summary, the results of this study demonstrate that depressed platelet function selectivity gains sensitivity to the aggregating agent, ADP following thyroxine replacement therapy. Although basal

cyclic AMP levels were not affected by thyroxine treatment, a marked reduction in the prostacyclin inhibitory system was observed. Depressed platelet sensitivity in the hypothyroid state may contribute to the easy bruising and bleeding tendancies and may explain the low incidence of myocardial infarction in these patients. The reports that thyroxine replacement therapy promotes undesirable vascular complications may in part be due to an overall improvement in metabolic rate and heart function. However, an improvement of platelet function and a reduced activity of the inhibitory feedback mechanism may be important contributory factors in the development of vascular complications. In addition, a concommitant decrease in fibrinolytic activity following thyroxine therapy (Bennet et al, 1967) may facilitate the formation of an occluding thrombus in the coronary arteries and may also have a pathogenic role in the genesis of vascular disorders.

It is likely that several factors may contribute to the various complications associated with thyroxine replacement therapy, but in light of these studies, it may be valuable to consider the addition of an anti-platelet drug to the therapeutic regime.

Chapter 7

General Discussion

The experiments described in this thesis have been concerned with the actions of stable analogues of several arachidonic acid metabolites. The synthetic endoperoxide and thromboxane mimetics have the same intrinsic biological activity as the parent compound and investigation of their biological actions has led to a better understanding of vascular haemostasis, including platelet function, in addition to stimulating new ideas in the development of future anti-thrombotic therapy.

Thromboxane A_2 has been implicated as the mediator of a variety of circulatory and cardiopulmonary disease states, including myocardial ischaemia (Lefer, 1982), some types of angina (Lewy et al, 1979, 1980), circulatory shock including traumatic and endotoxic shock (Lefer, 1983) and atherosclerosis (Mehta and Mehta, 1981). In addition platelets from diabetics display an increased release of thromboxane and their blood vessels have been found to have a decreased production of prostacyclin (Harrison et al, 1978; Johnson et al, 1978).

A group of drugs which have been widely used to reduce the deleterious effects of thromboxane A_2 are the non-steroidal anti-inflammatory drugs (NSAIDs, e.g. aspirin). They may do so by interfering with prostaglandin and thromboxane biosynthesis and hence reducing the tendency for platelets to aggregate.

However, it has been demonstrated that aspirin, whilst inhibiting thromboxane in platelets, also inhibited the synthesis prostacyclin to a similar degree in vascular tissue (Weskler et al,

1983; Preston et al, 1981). The "aspirin dilemma" emerged as a consequence (Moncada & Vane, 1979, Marcus, 1977) and the simultaneous inhibition of prostacyclin and thromboxane synthesis was considered a possible reason for the disappointing results of clinical trials of high dose aspirin therapy (de Gaetano et al, 1982). It was proposed that if certain aspirin dose regimens were devised it may be possible to inhibit platelet thromboxane synthesis without interfering with prostacyclin synthesis in vascular tissue (platelet cyclo-oxygenase was believed to be more sensitive than vascular cyclo-oxygenase to aspirin inhibition). Unfortunately, studies with cultured endothelial cells, laboratory animals and man have not completely dissociated the two effects (de Gaetano et al, 1982). Perhaps it should be considered, that the effects of these drugs may vary in different pathological conditions in which platelet and/or endothelial behaviour may be affected. Enhanced platelet turnover and/or endothelial damage may decrease the effectiveness and/or selectivity of aspirin.

Another approach considered in the design of anti-thrombotic therapy was the development of thromboxane synthetase inhibitors. Theoretically a selective inhibitor of thromboxane synthetase should prove to be a superior anti-thrombotic agent to aspirin and other NSAIDs, since it would allow continued prostacyclin formation by the vessel walls and other cells. Endoperoxides released from these tissues or those released from platelets may be utilized. Imidazole, one of the first such agents was found to inhibit the thromboxane synthetase enzyme at doses that had no effect on the cyclo-oxygenase enzyme (Moncada et al, 1977A). Thromboxane

synthetase inhibitors have indeed been widely studied in a variety of disease states with some degree of success (Lewis and Tyler, 1983). However, one drawback of thromboxane synthetase inhibitors is that they do not block the effects of the endoperoxides, PGG_2 and PGH_2 which have the same intrinsic activity as thromboxane. This is an important consideration since redirection of prostaglandin endoperoxides metabolism to prostacyclin which has been reported (Blackwell et al, 1978) will only occur at the site of the platelet vessel wall interaction if the prostacyclin synthesizing capacity is intact (Davies & Menys, 1983). If the vessel wall is damaged or obstructed by an atheromatous plaque, the prostacyclin synthetase enzyme may not utilize the platelet endoperoxides under these conditions. Furthermore, other redirected metabolites, particularly PGD_2 and $PGF_{2\alpha}$ may contribute to vasoconstrictor or cardiopessent effects. The future of thromboxane synthetase inhibitors as therapeutically useful drugs is dependent upon the maintenance of prostacyclin formation and a role for it in the preservation of endothelial thromboresistance.

The problems encountered with the thromboxane synthetase inhibitors may be overcome by the use of thromboxane receptor antagonists, which specifically act at the receptor level on the platelet or vascular tissue. Thromboxane receptor antagonists differ from thromboxane synthetase inhibitors in several ways and may be of importance 'in vivo' to combat the deleterious effects of thromboxane. The primary advantage of thromboxane receptor antagonists is that they antagonise the endoperoxides and thromboxane A_2 directly at the receptor level. The use of thromboxane receptor

antagonists does not lead to an increase in the synthesis of the classical prostaglandins (e.g. $PGF_{2\alpha}$) which may not be beneficial since they could constrict blood vessels. Furthermore, thromboxane receptor antagonists would protect the receptors from the effects of continually produced thromboxane and possibly be of greater therapeutic value once the disease process has started. Furthermore, antagonists are not likely to alter the bleeding time which is significantly increased with enzyme inhibitors.

Thromboxane receptor antagonists therefore have some theoretical advantages over other drugs and may be of value in circulatory disease states (i.e. circulatory shock, myocardial ischaemia, and other heamostatic disorders). Indeed preliminary data has indicated that thromboxane receptor antagonism may be an effective mode of therapy in circulatory shock, whereby thromboxane A_2 is thought to mediate the pathogenesis of the shock state by contributing to splanchnic ischaemia (Lefer et al, 1979, 1980).

A definitive characterisation of thromboxane receptor subtypes on platelets and the vasculature would perhaps allow the development of drugs exhibiting selectivity in their action, either at the platelet or on the vasculature and may improve the therapeutic usefulness of such thromboxane antagonists.

The first suggestion that platelets and vascular thromboxane receptors were different was based on early studies using the 'first generation thromboxane antagonists', PTA_2 and CTA_2 (Nicolaou et al, 1979). As mentioned previously in chapter 4, these compounds have

since been found to exert functional antagonism possibly through elevation in cyclic AMP (Jones et al, 1984). These additional effects may contribute to the intra-species differences reported in thromboxane sensitivity and the differences between tissues and platelets. If indeed these compounds are also acting at prostacyclin receptors, these receptors may also exhibit differential sensitivity between tissues, platelets and species. The differences observed with these compounds, may therefore not be solely due to thromboxane receptor differences.

In this instance, the use of prostacyclin receptor antagonists would have the potential to eliminate any prostacyclin-like effects and therefore allow the nature of these differences to be elucidated. Although prostacyclin would have no therapeutic value, they would have potential as pharmacological tools.

It would appear from studies in this thesis, that there are species differences in the sensitivity of some thromboxane sensitive-systems with respect to antagonist activity, yet similar in sensitivity to agonists. The differences in antagonist sensitivity between many vascular and platelet systems are small in comparison to the intra-species differences; the affinity of antagonists for the rabbit thromboxane receptor on platelets and the vasculature are very similar, but somewhat different from the affinities found on human and rat platelets. Similarly there are little differences in the antagonist affinities found on tissues such as guinea-pig trachea, dog saphenous vein and platelets from human and rat blood. Presumably the agonist binding site is similar in rat, rabbit and

human platelets and possibly to those on the vasculature. The difference in antagonist binding affinity may be a product of evolution, found only in certain species. Its significance however from an evolutionary (survival) point of view is not clear.

Owing to conflicting evidence put forward in other studies (Saussy et al, 1985, Mais et al, 1985B), there is a need for further definitive studies to characterise the nature of these underlying differences. It may be possible to decide whether there is a clear cut separation of these receptors based on cell types or whether there are different mixtures of receptors in differing ratios within each cell type.

Although the pharmacological approach to the characterisation of receptors from differing tissues or organs has its limitations in the interpretation of results, it has proven useful and provided enough information for the initial characterisation of receptors in many cases (e.g. B_1 & B_2 adrenergic and H_1 and H_2-histaminergic receptors). Subsequent 'in vitro' radioligand binding assays may in fact provide a more definitive conclusion as to whether thromboxane receptor hetereogeneity exists. Radioligand binding studies may provide the answers as to whether there are differences in affinity, receptor number or perhaps distribution of receptors. As already discussed, the rabbit platelet receptor and possibly the rabbit vascular thromboxane receptor have a somewhat lower affinity for thromboxane antagonists. In order to achieve sufficient binding of a labelled ligand to attain a displacement curve, ligand concentrations of 10-100μM would be necessary. Unless the

radioligand solution could be diluted with cold labelled ligand, excess quantities of radioligand would need to be used in ligand binding studies on rabbit platelets or vascular tissue and such exercises would prove to be extremely costly.

If the thromboxane/endoperoxide receptor represents a protein complex embedded in a lipid domain in the membrane, perhaps a subtle conformational alteration of the protein structure may be reflected by the differences in affinities observed between the receptors. If the antagonist action is dependent upon the conformational size and/or lipophilicity/hydrophilicity of the molecule, perhaps only a subtle conformational alteration at the rabbit thromboxane receptor may decrease the accessibility of the antagonists.

The recent solubilization of an endoperoxide/thromboxane A_2 binding site (Saussy et al, 1985) has demonstrated such a site in an active form. Antagonists were readily displayed by other agents. The dissociation constant values for a series of analogues were similar to those determined from binding studies and pharmacological experiments on platelets. The binding site was of protein nature with a molecular mass of about 200 kDaltons. Purification of this binding unit and analysis of the structure may in fact open up another approach whereby the nature of thromboxane receptor differences may be examined at the molecular level.

Other alternatives for anti-platelet and anti-thrombotic drugs may be sought, particularly in conditions where thromboxane may not be the sole mediator of the deleterious effects. The development of

orally active prostacyclin analogues with a long duration of action may provide the answer for drugs to control 'in vivo' platelet aggregation and thromboembolic disorders and which would be superior to enzyme inhibitors which are generally less selective. However, since prostacyclin and its derivatives exert potent vasodilator effects as well as anti-platelet action, long-term vasodepressor actions may not be without other side effects, possibly on the cardiovascular system. The definitive separation of platelet and vascular actions in an active synthetic prostacyclin analogue both for use as a research tool and as a clinically useful agent would be the ultimate objective in the development of prostacyclin mimetics.

However, until the pharmacokinetic parameters of these analogues are fully elucidated and some mechanism of controlling the distribution of the analogues from the blood to the various tissue compartments is developed, metabolically stable prostacyclin analogues with long duration of action 'in vivo' will remain to be identified. Although there is some evidence from experimental studies of selectivity on platelets distinct from the vasodepressor action of prostacyclin, there is as yet no stable analogue that has a highly selective action on platelets 'in vivo' (Karim et al, 1981).

Recently the development of compounds which act both as prostacyclin mimetics and thromboxane antagonists (EP035, EP057, Armstrong et al, 1986) offers another promising approach for therapeutic measures against thromboxane action. These types of compounds may prove to be therapeutically useful since in addition to specifically antagonising TXA_2, they would eliminate any

vasoconstriction or residual platelet aggregation induced by the prostaglandin endoperoxides and also have direct PGI_2-like anti-aggregatory activity.

Prostacyclin by increasing cyclic AMP prevents platelet aggregation induced by all known agonists as well as disaggregating platelets 'in vitro' (Gryglewski et al, 1976). 'In vivo' multiple agonists are likely to act in concert. In a syndrome of platelet mediated vascular occlusion, inhibition of thromboxane action alone may not be completely effective. Thus compounds such as EP035 and EP057 may have greater therapeutic potential in situations where there is an increased thromboxane production as well as an increase in mediators of aggregation and vasoconstriction which are non-thromboxane.

Before the discovery of prostacyclin, it was suggested that dietary dihomo-γ-linolenic acid, the precursor of prostaglandins of the '1' series (e.g. PGG_1, PGE_1 and TXA_1) could be employed as a measure in the prevention of thrombosis. PGG_1 and thromboxane A_1 have no pro-aggregatory effects and in fact PGE_1 has an anti-aggregatory activity. However, the use of dihomo-γ-linoleic acid is possibly not the most rational approach for the prevention of thrombosis since the endoperoxides PGG_1 and PGH_1 are not substrates for prostacyclin synthesis. An accumulation of these substances or dihomo-γ-linolenic acid could adversely affect the prostacyclin protective mechanism (Willis et al, 1974). An alternative means of dietary manipulation in favour of decreasing aggregatory/vasoconstrictor compounds without affecting antiaggregatory measures to any great

extent could be achieved by incorporating the polyunsaturated fatty acid, eicosapentaenoic acid (E.P.A.) into the diet. Eicosapentanoic acid has a higher degree of unsaturation and metabolism of this fatty acid gives rise to prostaglandins of the '3' series. When vascular tissue is incubated with eicosapentaenoic acid, an anti-aggregatory substance is released namely prostacyclin I_3 (Gryglewski et al, 1979). Synthetic prostacyclin I_3 is as potent in anti-aggregatory activity as prostacyclin. In contrast, thromboxane A_3 has weaker pro-aggregatory activity than thromboxane A_2 (Raz et al, 1979). Furthermore, it was found in studies in this thesis, that eicosapentanoic acid itself has an anti-platelet action. Gryglewski and co-workers (1979) have also shown that it inhibits 'in vitro' platelet aggregation induced by ADP, collagen and arachidonic acid. 'In vivo' eicosapentaenoic acid is incorporated into the platelet phospholipids replacing some of the arachidonic acid. It may exert an anti-platelet effect by either competing with arachidonic acid for the cyclo-oxygenase and lipoxygenase enzymes (Culp et al, 1980) or by being converted to the less pro-aggregatory compounds, PGH_3 and TXA_3 (Gryglewski et al, 1979).

Fish and fish oils are rich in eicosapentaenoic acid and studies have shown that diets high in eicosapentanoic acid sources alters the ratio of arachidonic/eicosapentaenoic acid present in membrane phospholipids (Dyerberg and Bang, 1979; Black et al, 1979; Seiss et al, 1980). In fact, the incidence of thrombosis and subsequent infarct size was found to be reduced in dogs fed on a fish oil diet (Culp et al, 1980). The high fish diet of Eskimos may explain why this group have a low incidence of acute myocardial infarction, low

blood cholesterol levels and an increased tendancy to bleed (Dyerberg and Bang, 1979).

Thus with regards to dietary manipulation either by supplementation or by dietary change another possible therapeutic approach in the treatment of thrombotic disorders or as a prophylactic measure is made available. Perhaps over the next few years, the development of these ideas will provide, more efficient and safer therapies.

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Originals

Increased platelet thromboxane receptor sensitivity in diabetic patients with proliferative retinopathy

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Summary. Platelet aggregation to collagen in 12 Type 1 (insulin-dependent) diabetic patients with background retinopathy and 12 Type 1 diabetic patients with proliferative retinopathy was compared with an age- and sex-matched control group. An analogue of prostaglandin H_2, 11,9 epoxymethano-prostaglandin H_2, which directly stimulates thromboxane receptors, and EP 092, which is a competitive thromboxane A_2 receptor antagonist, were used to investigate changes at platelet thromboxane receptor level in these groups. The concentration of collagen (EC_{50}) required to give 50% of maximum aggregation did not differ between the two diabetic groups and the control group. However, platelets from the proliferative retinopathy group were significantly more sensitive to the thromboxane mimetic (11,9 epoxymethano-prostaglandin H_2) ($p < 0.005$) than the background retinopathy and control groups. This change may be a factor in the development of proliferative retinopathy.

Key words. Platelets, thromboxane sensitivity, cAMP, proliferative retinopathy.

Previous studies in diabetes mellitus have demonstrated abnormalities of platelet function such as exaggerated response to aggregating agents [1] and refractoriness to inhibiting prostaglandins (PG) [2, 3]. Hyperaggregation is reported to be associated with poor glycaemic control [4], increasing age [5], and the presence of micro- and macrovascular changes [6, 7]. A number of circulatory substances relevant to coagulation, including lipids [8], fibrinogen [9] and von Willebrand factor [10] have also been reported as abnormal in diabetic patients. However, particularly in the presence of vascular complications [11, 12, 13], the results have been inconsistent, perhaps because of the diverse methods used and the failure to obtain homogenous patient groups.

Where a possible mechanism for enhanced platelet aggregation has been investigated, the most consistent finding has been the increased production of aggregation-induced thromboxane A_2 (TXA_2) [14, 15]. TXA_2, which is the predominant metabolite of platelet arachidonic acid (AA), is a powerful platelet aggregator and vasoconstrictor [16, 17]. Most studies have employed AA itself or an agent such as collagen, which releases endogenous platelet AA, to demonstrate enhanced platelet aggregation. Since the production of TXA_2 is known to be increased, it cannot be determined from these studies [11, 12] whether improved receptor coupling or an increase in the number of thromboxane receptors may also contribute to this increased sensitivity.

The aims of this study were to confirm platelet hyperaggregability to collagen and to investigate the changes at receptor level in platelets from diabetic patients with established microangiopathy using an analogue of PGH_2, 11,9 epoxymethano PGH_2 (11,9-em PGH_2), which directly stimulates thromboxane receptors [18, 19], plus a competitive thromboxane receptor antagonist (EP 092) [20]. Young insulin-dependent diabetic patients who had background and proliferative retinopathy as well as a non-diabetic control group were selected in order to investigate the possible relationship between platelet function and susceptibility to proliferative retinopathy.

Subjects and methods

The patients studied were 24 Type 1 (insulin-dependent) diabetic patients, 12 with background and 12 with proliferative retinopathy. The patients were compared with 12 non-diabetic healthy volunteers. The three groups were comparable for age and sex, and the diabetic groups were matched for duration of diabetes. There was no significant difference between the two diabetic groups in blood glucose, HbA_1 or platelet counts at the time of the study. (Table 1). Classification of retinopathy was based on ophthalmoscopic and fluorescein angiographic appearances in all patients. In the background retinopathy group three patients had scattered microaneurysms and haemorrhages, seven patients had hard exudative changes and two patients had ischaemic preproliferative changes. None of the diabetic subjects were taking any medication other than insulin, all of the patients were

Table 1. Average age, duration of diabetes, plasma glucose, HbA_1 and platelet counts in control and diabetic retinopathy groups

Group	Number of Subjects	Average age (yrs)	Duration of diabetes (yrs)	Plasma glucose (mmol/l)	HbA_1 (%)	Platelet count ($\times 10^9$/l)
Control	12 (6F, 6M)	26.7 ± 1.3	-	4.6 ± 0.3	7.6 ± 0.3	274 ± 11
Background Retinopathy	12 (6F, 6M)	27.6 ± 0.9	15.2 ± 1.1 NS	10.5 ± 1.0 NS	11.8 ± 0.7 NS	257 ± 16 NS
Proliferative Retinopathy	12 (6F, 6M)[a]	27.0 ± 1.5[a]	14.6 ± 1.4[b]	11.6 ± 1.7[b]	12.6 ± 0.7[b]	245 ± 12[b]

Results expressed as mean ± SEM. [a] NS between the three groups; [b] NS between the diabetic groups

Table 2. Results of aggregation EC_{50} and dose ratio for collagen and 11,9 emPGH_2 alone and in the presence of EP 092 (0.25 μmol/l) and Froben (10 μmol/l). Results expressed as mean ± SEM

Group	Collagen EC_5^0 (μg/ml)	Collagen + EP 092 EC_{50} (μg/ml)	Dose Ratio	Collagen + Froben EC_{50} (μg/ml)	Dose Ratio	11,9 emPGH_2 EC_{50} (ng/ml)	11,9 emPGH_2 + EP 092 EC_{50} (ng/ml)	Dose Ratio	11,9 emPGH_2 + Froben EC_{50} (ng/ml)	Dose Ratio
Control	0.8 ± 0.1	1.9 ± 0.3	2.4 ± 0.2	8.3 ± 1.9	9.8 ± 2.0	99.4 ± 10.0	115.3 ± 5.2	1.7 ± 0.1	124.7 ± 9.4	1.31 ± 0.1
Background retinopathy	0.74 ± 0.1	1.8 ± 0.3	2.7 ± 0.5	6.6 ± 1.6	10.8 ± 3.4	85.4 ± 11.5	193.8 ± 28.4	2.6 ± 0.6	120 ± 12.9	1.51 ± 0.4
Proliferative retinopathy	1.1 ± 0.2	1.9 ± 0.4	1.9 ± 0.2	4.9 ± 0.7	5.0 ± 0.5	63.5 ± 6.6	176.0 ± 20.2	3.15 ± 0.6	100.4 ± 8.8	1.62 ± 0.1
Significance	NS	NS	NS	NS	NS	$p < 0.005$	NS	$p < 0.005$	NS	NS

$p < 0.005$, significant difference between proliferative retinopathy group and background retinopathy group

clinically free of nephropathy (urine Albustix negative and plasma creatinine less than 150 μmol/l) and all had easily palpable peripheral pulses.

Blood (100 ml) was collected between 08.00 and 09.00 hours from fasted diabetic patients and control subjects into plastic centrifuge tubes containing acid-citrate dextrose (ACD) anticoagulant and centrifuged at 120 g for 20 min. The platelet-rich plasma (PRP) was then pre-incubated with [^{14}C] 5-hydroxytryptamine (final concentration 1 μg/ml) for 30 min at 37 °C. Platelet aggregation was studied using the photometric method of Born [21, 22]. Dose response curves for aggregation induced by collagen and 11,9-em PGH_2 were determined both in the presence and absence of a cyclooxygenase inhibitor, Froben (10 μmol/l), which eliminated the production of endogenous TXA_2 and allowed assessment of aggregation in the absence of the secondary aggregation wave. In addition, we measured the shift in dose-response curve for aggregation using a fixed concentration of a competitive TXA_2 receptor antagonist EP 092 (0.25 μmol/l). Each aliquot was warmed to 37 °C for 2 min prior to the addition of the aggregating agents. If used, Froben or EP 092 were added at time zero, 2 min before the addition of the aggregating agent.

Release of platelet dense granule constituents (5HT, ADP) were measured using the radiolabelled isotope [^{14}C] 5-hydroxytryptamine prelabelling technique [22]. Basal platelet cAMP and the rise in cAMP induced by PGE_1 (0.2 μmol/l) were measured by a protein binding assay [22, 23].

The glycosylated haemoglobin assay was carried out by electrophoresis using commercially available agar plates (Corning Medical, Halstead, UK) as described by Read et al. [24], the normal range being 6–8%.

11,9-em PGH_2 and PGE_1 were purchased from Upjohn, Kalamazoo, Mi, USA. Collagen was obtained from Diamed Diagnostics Ltd, Liverpool, England. EP 092 was synthesised in the laboratory of the Department of Pharmacology, University of Edinburgh. Flurbiprofen (Froben) was a gift from Boots Pharmaceuticals Ltd, Nottingham, England. [^{14}C] 5-hydroxytryptamine was purchased from Amersham, UK. All of the solutions were free of ethanol and all subsequent dilutions were made up in 0.9% saline.

Statistical analysis

Statistical analysis of the dose ratios was by Kruskal-Wallis one-way analysis of variance by ranks. Comparison between the groups for the cAMP results was by independent Student's t-test.

Results

There was no difference in the concentration of collagen (EC_{50}) required to give 50% of the maximum aggregation wave between the two diabetic groups and the control group (Table 2). Similarly, the thromboxane receptor antagonist EP 092 shifted all three curves to a similar extent, as shown by the new EC_{50} values in the presence of EP 092, and by the dose ratios (DR) ($DR = EC_{50}$ in the presence of EP 092/EC_{50} collagen alone). The cyclooxygenase inhibitor Froben produced a more marked shift of the control aggregation curves than observed with EP 092. Froben appeared to be slightly less effective in the proliferative retinopathy group, but this was not statistically significant.

There was, however, a significant difference between the three groups in the concentration of 11,9-em PGH_2 required to induce a 50% aggregation response. Less 11,9-em PGH_2 was required for the proliferative retinopathy group than for either the control or retinopathy groups ($p < 0.005$). Furthermore, EP 092 produced a significantly larger shift in the aggregation curve in the proliferative retinopathy group as judged by the dose

Table 3. Results of platelet release reaction in control and diabetic retinopathy groups due to collagen (2 μg/ml) and (250 ng/ml) 11,9 emPGH$_2$ alone and in the presence of EP 092 (0.25 μmol/l) and Froben (10 μmol/l)

Group	Collagen	Collagen + EP 092	Collagen + Froben	11,9 emPGH$_2$	11,9 emPGH$_2$ + EP 092	11,9 emPGH$_2$ + Froben
Control	58.8 ± 2.2	41.1 ± 3.6	19.8 ± 1.1	34.9 ± 1.0	30.9 ± 1.4	32.7 ± 1.3
Background retinopathy	51.8 ± 4.5	39.3 ± 3.9	21.6 ± 1.4	30.8 ± 2.9	26.7 ± 2.9	29.3 ± 2.3
Proliferative retinopathy	55.3 ± 5.7	30.7 ± 4.1	18.4 ± 2.4	32.8 ± 2.4	28.7 ± 3.4	30.1 ± 1.7

Results expressed as % total [^{14}C]-5-HT released/[^{14}C]-5-HT taken up by platelets at pre-incubation and given as mean ± SEM

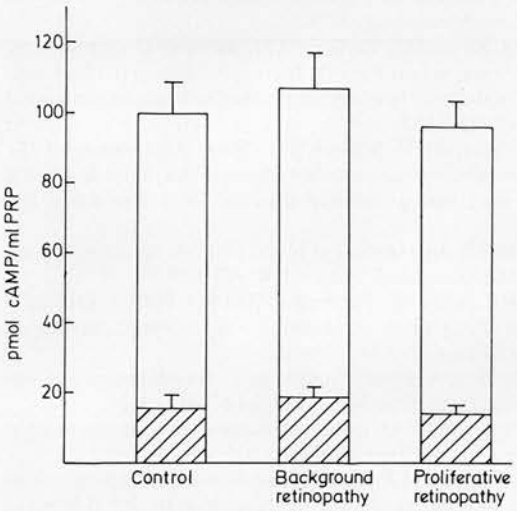

Fig. 1. Basal platelet cAMP levels and cAMP levels after exposure to prostaglandin E_1 (PGE_1) in control and diabetic retinopathy groups. Results given as mean ± SEM. $n = 12$ in all groups. ▨ Basal level of cAMP; □ cAMP level after exposure to prostaglandin E_1. PRP = platelet rich plasma

ratio when compared with the other two groups ($p < 0.005$). In the presence of Froben no difference was found between the three groups. (Table 2)

5-Hydroxytryptamine release in response to both collagen and 11,9-em PGH_2 showed no difference between the three groups (Table 3).

There was no difference in the basal levels of cAMP found in platelets from the three groups or in the rise in cAMP induced by PGE_1 (Fig. 1).

Discussion

This study demonstrated that platelets from Type 1 diabetic patients with proliferative retinopathy are more sensitive to 11,9-em PGH_2 than platelets from Type 1 diabetic patients with background retinopathy and control subjects. However, unlike previous studies in diabetic patients [15, 26–28], we did not find increased platelet sensitivity to collagen, whether sensitivity was measured as platelet aggregation or as 5-hydroxytryptamine release. In non-diabetic subjects endogenous PGH_2 and TXA_2 production are the major components of collagen-induced aggregation [29]. The aggregation EC_{50} for collagen was similar for the two diabetic and control groups, with the reversible thromboxane antagonist EP 092 producing a similar shift of the aggregation wave in each group. The cyclooxygenase inhibitor Froben appeared to be slightly less effective in the proliferative group than in the background retinopathy and control groups, but this did not achieve statistical significance. The slight decrease in the efficacy of Froben in this group is consistent with increased thromboxane synthesis in response to collagen without alteration in the aggregation EC_{50} level. From these observations it would appear that the mechanism for collagen-induced aggregation is similar in both diabetic patients and control subjects.

Age is an important determinant of platelet function in both non-diabetic subjects and diabetic patients [5]. During recruitment to our study we paid particular attention to careful age restriction and comparability. This may account for the apparent discrepancy in platelet collagen sensitivity in diabetic patients in this study compared with other studies [15, 26–28].

In contrast, platelets from the diabetic group with proliferative retinopathy were more responsive to the thromboxane mimetic 11,9-em PGH_2. This increase in sensitivity was reduced in the presence of the cyclooxygenase inhibitor, Froben. In non-diabetic subjects the major mechanism of the action of the mimetic does not involve activation of the thromboxane generating system [16, 17]. It would appear, therefore, that the increased sensitivity is not a direct effect of the 11,9-em PGH_2 on the receptor. The mechanism of increased sensitivity in the proliferative retinopathy group remains undefined; however, it may be due to increased endogenous thromboxane production during secondary aggregation.

There was no difference between either the basal level of platelet cAMP levels or the increased cAMP level after exposure to PGE_1 between diabetic patients and control subjects. This is in agreement with a recent study of PGI_1 binding to platelets [31] and activation of adenylate cyclase, but in sharp contrast to that of Lagarde et al. [32], where lower resting levels of cAMP and a diminished response to inhibiting prostaglandins were

reported. The likely explanation is that in our study and that by Shepherd et al. [31] the subjects were young and age-matched, whereas in the report by Lagarde et al. [32] the ranges of age, duration of diabetes and vascular complications were very wide.

In conclusion, platelets from young Type 1 diabetic patients with microvascular disease in the form of background and proliferative retinopathy are similar to platelets from normal subjects in their response to collagen-induced aggregation. In contrast, platelets from diabetic patients with proliferative retinopathy demonstrate increased sensitivity to a thromboxane mimetic; this may be a factor in the development of proliferative retinopathy.

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